Molecular and metabolic asspects of succinate thiokinase

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MOLECULAR AND METABOLIC ASPECTS OF SUCCINATE THIOKINASE

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for the degree of Ph.D.

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Proverbs 25[2]

"It is the glory of God to conceal a matter;
to search out a matter is the glory of kings".

Psalm 24[1]

"The earth is the Lord's, and everything in it,
the world, and all who live in it".

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ABSTRACT

In the last twenty years, a great deal of attention has been focused upon understanding the molecular mechanisms of both the prokaryotic and eukaryotic succinate thiokinase (STK) enzymes. However, relatively little is known concerning the significance of the diversity of nucleotide specificity displayed by STK throughout Nature.

This thesis presents evidence for the existence of two distinct STK enzymes in animal tissues - one (G-STK) linked to guanine nucleotides and the other (A-STK) to adenine nucleotides. This finding prompted re-examination of prokaryotic organisms and lead to the discovery of an STK of novel nucleotide specificity for inosine nucleotides.

Investigations into the possible physiological roles of the distinct STKs within animal tissues involved induction of physiological dysfunction (diabetes and porphyria) together with comparative studies on different organisms. These findings support the proposal that the two STKs are responsible for catalysing the succinate thiokinase reaction in opposite directions. It appears that A-STK may be involved with the energy generation of the citric acid cycle, in the direction of succinyl-CoA hydrolysis, whereas G-STK is responsible for providing succinyl-CoA for ketone body metabolism and haem biosynthesis, in the direction of succinyl-CoA formation.

The physiological studies have been supported by enzyme purification and molecular and kinetic characterizations of the STKs.

ABBREVIATIONS

The majority of abbreviations are those recommended in the Biochemical Society publication "Policy of the Journal and Instructions to Authors", Biochem. J. (1988) 249, 1-20.

Non-Standard Abbreviations:

DTNB	5,5'-dithiobis-(2-nitrobenzoic acid)
DTT	dithiothreitol
$K_{(INACT.)}$	First-order rate constant of thermal inactivation (S^{-1})
MET8	20 mM Tris-HCl + 1 mM EDTA + 2 mM $MgCl_2$ buffer, pH 8·0
Pi	inorganic phosphate
TEA	Triethanolamine buffer
TPP	Thiamine pyrophosphate
TEMED	N,N,N',N'-Tetramethylethylenediamine

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Finally, a special thankyou to my parents for their encouragement and support and to Liz for her endless patience and constant love.

CHAPTER 1

INTRODUCTION

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1·1 Historical Perspectives

Discovery of the citric acid cycle

The citric acid cycle, with few exceptions, is probably the most important pathway or unit of metabolism in aerobic organisms. The final steps, which completed the citric acid cycle, were elucidated by Krebs & Johnson (1937), building on the work of Szent-Györgyi and Martius & Knoop (Krebs, 1970). Krebs correctly identified that oxidation of citrate yielded oxaloacetate, which together with a proposed "unknown triose" derived from carbohydrate metabolism, reacted to regenerate citrate and so complete the cyclic set of reactions, termed by Krebs the "citric acid cycle". This discovery, following soon after the proposal of the urea cycle (Krebs & Henseleit, 1932), established metabolic cycling as an important concept in biochemical pathways. The citric acid cycle was soon accepted and recognised as the terminal pathway for the oxidation of 'fuel' molecules (carbohydrates, fats and even proteins) in all respiring animals, plants and aerobic micro-organisms.

After clarification of the energy-yielding catabolic role of the cycle, it was later proposed (Krebs *et al.*, 1952) that it may also possess an anabolic function,

providing intermediates for cellular biosynthetic purposes. Subsequent studies rapidly confirmed this proposal (Ajl, 1958) establishing the dual roles of the citric acid cycle, in supplying both energy and cell components. The catabolic and anabolic roles of the citric acid cycle are illustrated in Figures 1 and 2, respectively.

Discovery of Coenzyme A and enzyme 'P'

Not until the discovery of Coenzyme A (CoA) by Lipmann(1945), did the detailed schemes of the citric acid cycle and fatty acid metabolism emerge. In the meantime it had been suggested that an "activated acetate" (Lynen, 1942), rather than an "unknown triose" (Krebs, 1943) was introduced into the citric acid cycle. Eventually, six years after the discovery of CoA, acetyl-CoA was demonstrated to be the "activated acetate" (Lynen *et al.*, 1951) involved in fatty acid metabolism and in the citric acid cycle, reacting with oxaloacetate to form citrate and CoA.

Conversion of oxoglutarate to succinate + CO_2 had been shown to be dependent upon the presence of CoA and NAD^+ (Korkes *et al.*, 1952). The oxidative decarboxylation of oxoglutarate was soon recognised to be analogous to the oxidative decarboxylation of pyruvate, with resultant acetyl-CoA + CO_2 production. Therefore it was predicted that a similar reaction product would result, that is the acyl-CoA derivative, succinyl-CoA.

Figure 1. Catabolic roles of citric acid cycle

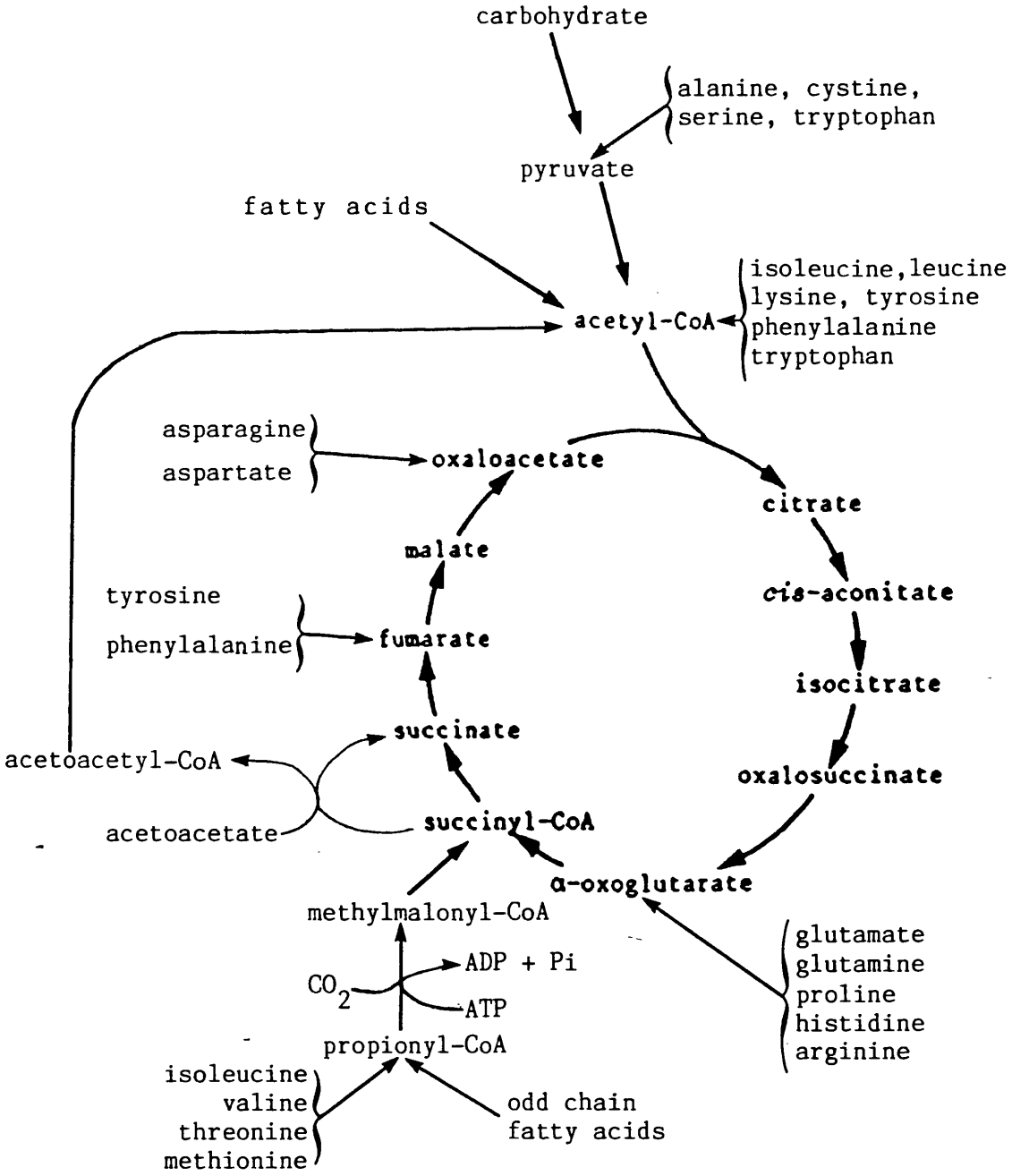
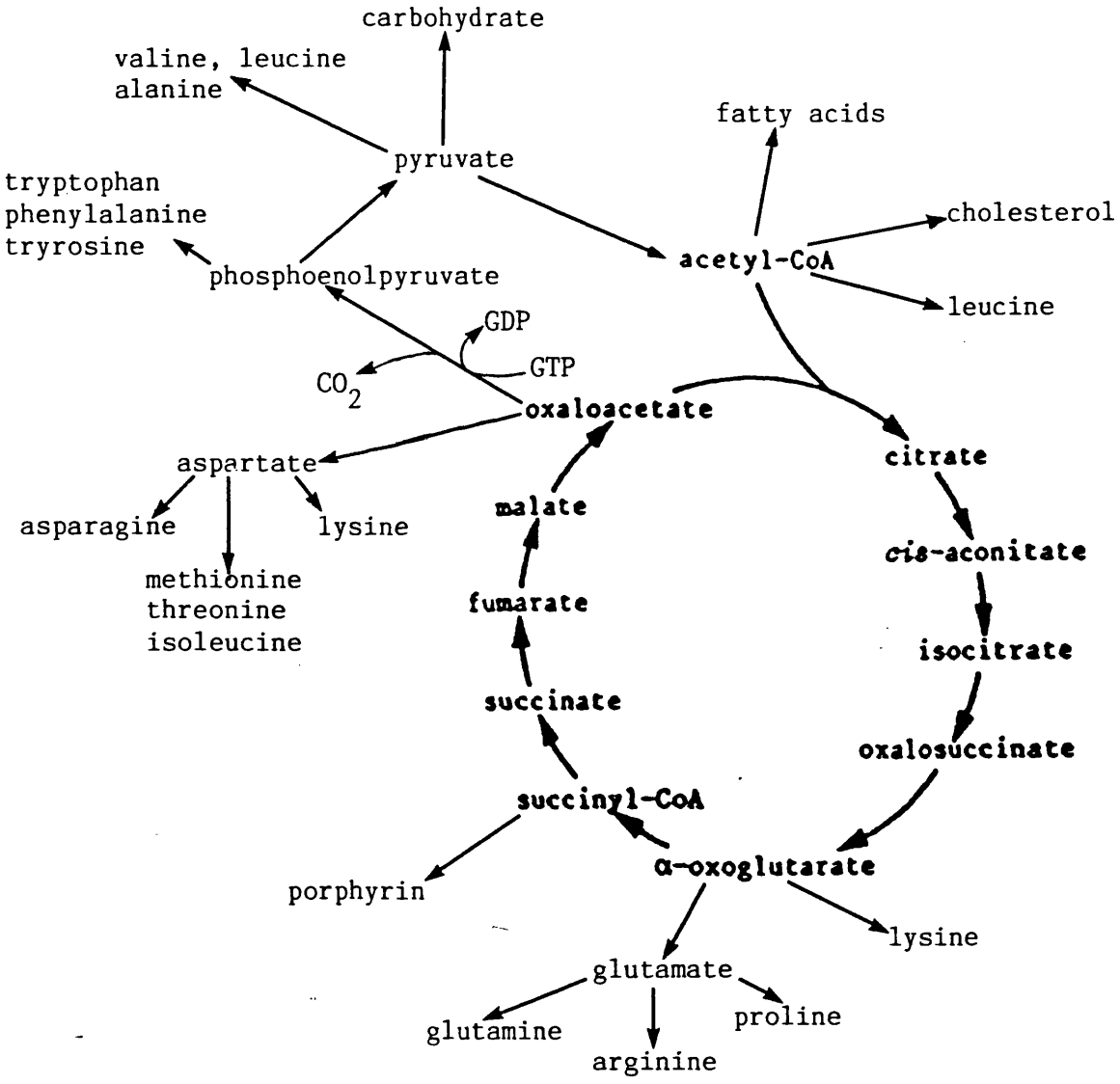


Figure 2. Anabolic roles of citric acid cycle



It was assumed that an enzyme termed succinyl-CoA deacylase then hydrolysed succinyl-CoA to succinate + CoA (Gergely *et al.*, 1952).

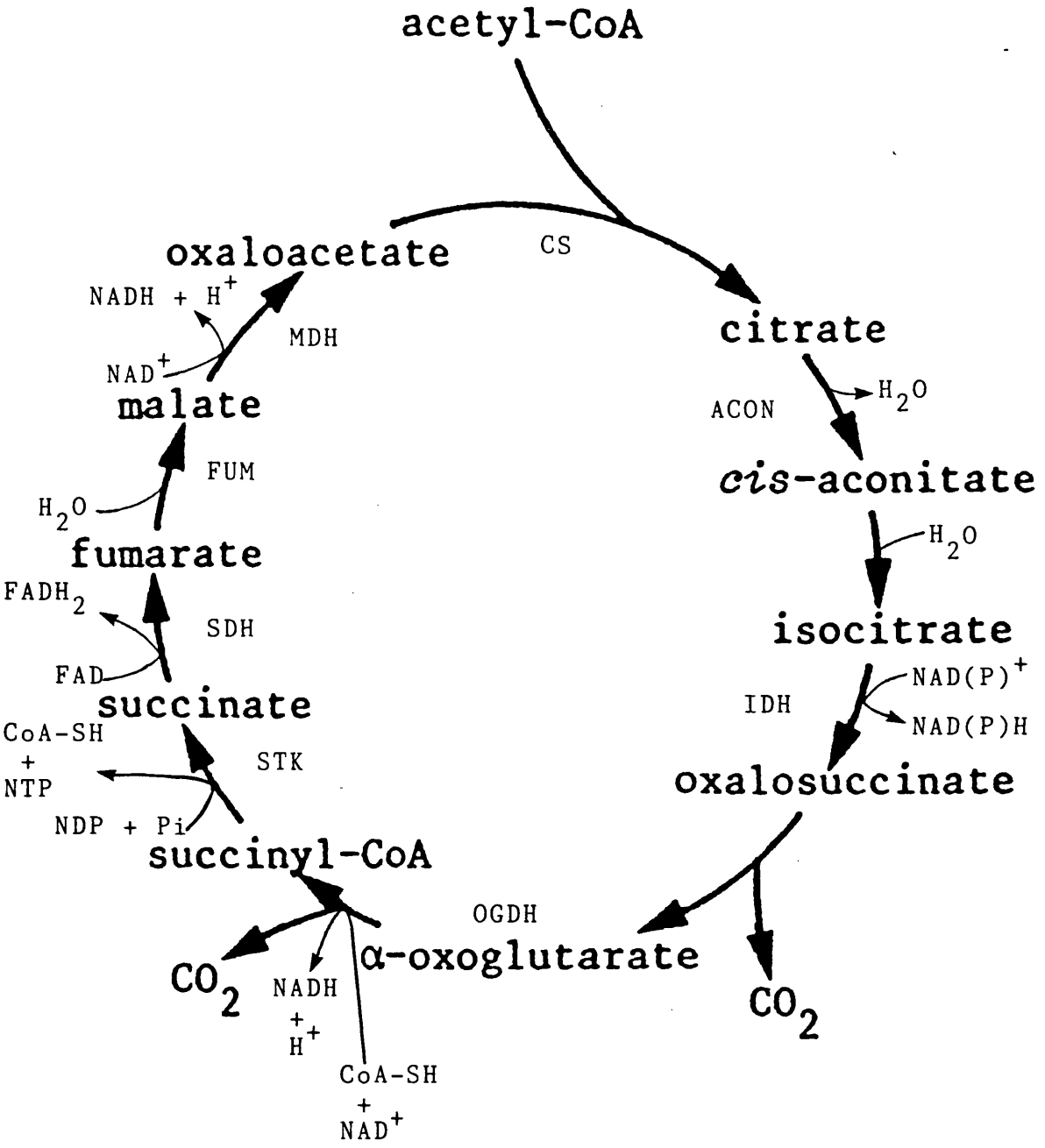
However, conversion of oxoglutarate to succinate + CO_2 was known to be coupled to phosphorylation (Hunter, 1951), Kaufman (1951) having also shown that soluble pig heart preparations catalysed the liberation of inorganic phosphate from ATP, only when both CoA + succinate were present. Further work (Kaufman, 1953) identified a "phosphorylating enzyme" as being responsible. Subsequently, Sanadi and co-workers (Sanadi *et al.*, 1956) were able to demonstrate that the enzyme from pig heart and kidney, when freed from nucleoside diphosphokinase (NDPK) activity, was specific for guanine nucleotides (GDP/GTP). Thus the substrate level phosphorylation step of the citric acid cycle (see Figure 3) was established.

succinyl-CoA + NDP + Pi $\rightleftharpoons$ succinate + CoA + NTP

(where NDP/NTP represent nucleoside diphosphate & triphosphate)

The enzyme responsible for the above reaction has been variously termed phosphorylating ('P') enzyme (Kaufman *et al.*, 1953); GDP phosphorylating enzyme (Sanadi *et al.*, 1955); succinyl-CoA synthetase (Smith *et al.*, 1957); and succinic or succinate thiokinase (Hager, 1957). The reaction was soon demonstrated to be freely reversible

Figure 3. The citric acid cycle



with an equilibrium constant of 3·7 at 20°c (Kaufman & Alivisatos, 1955). This value has recently been revised, after a careful study at physiological ionic strength, pH ranges and free [Mg^{2+}], to 3·02 ± 0·07 and 1·29 ± 0·05 at 25 and 38°c respectively (Lynn & Guynn, 1978). It is therefore apparent that, in the forward direction, succinate thiokinase (STK) may participate in the citric acid cycle in an energy conserving step, whereas in the reverse direction, it may control the provision of succinyl-CoA for biosynthetic purposes (see Section 1·7).

Interestingly, citrate synthase has in common with STK the utilization of an acyl-CoA derivative as a substrate. STK couples the energy of succinyl-CoA hydrolysis to the phosphorylation of a nucleoside diphosphate, whereas citrate synthase uses the energy available on the hydrolysis of acetyl-CoA to form a covalent carbon-carbon bond of the citric acid molecule.

1·2 Location & Regulation of citric acid cycle

Kennedy and Lehininger (1949) established that, in mammals, the cellular location of the cycle is the mitochondrion. Further work confirmed this, but has identified the occurence of extra-mitochondrial isoenzymes of the cycle, e.g. NADP-linked isocitrate dehydrogenase (Hogeboom & Schneider, 1950); malate dehydrogenase (Roodyn *et al.*, 1962) and, more recently, citrate synthase (Rickey & Lewin, 1986).

To enable the citric acid cycle to display both anabolic and catabolic roles, a number of ancillary enzyme systems exist, supplying or removing cycle intermediates. These are known as 'anaplerotic' and 'cataplerotic' reactions respectively. Anaplerotic mechanisms comprise pyruvate or propionate carboxylations (Davis *et al.*, 1980), phosphoenolpyruvate carboxykinase (Penhkurinen, 1984), coupled transamination reactions of aspartate and alaine aminotransferases (Safer & Williamson, 1973), glutamate dehydrogenase and the purine nucleotide cycle (Aragon & Lowensteim, 1980). Cataplerotic reactions include the intra-mitochondrial NAD(P)- and NADP-linked malic enzyme, oxaloacetate decarboxylase, coupled transaminations and phosphoenolpyruvate carboxykinase (Penhkurinen, 1984).

Whilst the chemistry of the citric acid cycle and related reactions are well established, the precise manner in which the flux through these pathways is regulated is still subject to much study and debate. Substrate availability and intra-mitochondrial parameters such as the ATP/ADP, acetyl-CoA/CoA-SH, succinyl-CoA/CoA-SH and acetyl-CoA/succinyl-CoA ratios are all considered important effects. However, the key energy-linked regulatory factor determining citric acid cycle flux is thought to be the mitochondrial free NAD^+/NADH ratio (Williamson, 1980). Considerable evidence also suggests that Ca^{2+} may play a crucial role in the activation of pyruvate, NAD-isocitrate and 2-oxoglutarate dehydrogenases and so increase cycle flux (McCormak & Denton, 1986).

Clearly, the multiple and varied metabolic constraints placed upon tissues and organisms, require a complex regulation of individual enzymes with the citric acid cycle.

1·3 Succinate Thiokinase - Molecular size and Nucleotide substrate diversity

The STKs which have received the most attention are those from *Escherichia coli* and pig heart (Nishimura, 1986). A marked difference in the molecular sizes of the two enzymes has been found. The *E. coli* enzyme is known to be an $\alpha_2\beta_2$ tetramer of Mr ~ 140,000 (Ramaley *et al.*, 1967), whereas the pig heart enzyme is an $\alpha\beta$ dimer of Mr ~ 75,000 (Brownie & Bridger, 1972).

Prompted by this difference, as well as the molecular size diversity already seen between citrate synthases from various prokaryotic and eukaryotic sources (Weitzman, 1981), an examination was undertaken of the sizes of STKs in a wide range of organisms (Weitzman & Kinghorn, 1978) (see Figure 4). This was achieved by gel filtration of cell-free extracts through Sephadex G-200 using lactate dehydrogenase (LDH) as a marker protein (Mr ~ 140,000). STKs of a similar molecular weight to the *E. coli* enzyme were eluted from the Sephadex column slightly ahead of LDH and were termed 'large', whereas STKs with molecular weights similar to the pig heart enzyme were eluted much later than LDH and were termed 'small'. Remarkably it was discovered that the 'large' STK is found only in Gram-

Figure 4 Molecular sizes of STK

"Large" enzyme	"Small" enzyme
Acinetobacter spp.	*Bacillus megaterium*
Aeromonas formicans	*Bacillus stearothermophilus*
Brevibacterium leucinophagum	*Brevibacterium linens*
Escherichia coli	*Corynebacterium rubrum*
Halobacterium salinarium	
Herellea vaginicola	Baker's yeast
Klebsiella (Aerobacter) aerogenes	Cauliflower mitochondria
Pseudomonas spp.	Wheat germ
Rhodopseudomonas sphaeroides	
Thermus aquaticus	Pig heart
Xanthomonas hyacinthi	Pig liver
Cyanobacteria	
Anabaenopsis circularis	
Anacystis nidulans	
Aphanocapsa 6714	
Chlorogloea fritschii	
Gloeocapsa alpicola	
Nostoc sp. E	
Plectonema boryanum	

negative bacteria, the 'small' STK being associated with Gram-positive bacteria and eukaryotic organisms. This work provides a striking correlation between the incidence of 'large' and 'small' STKs and that of 'large' and 'small' citrate synthases in a wide spectrum of organisms.

Another intriguing facet of STK diversity is their specificity for the nucleotide substrate. The mammalian enzyme was originally found to be specific for guanine nucleotides (GDP/GTP) or inosine nucleotides (IDP/ITP) (Sanadi *et al.*, 1956) and this is still held to be true. The enzyme from *E. coli* was reported to be specific for adenine nucleotides (ADP/ATP) (Robinson *et al.*, 1969), with plant and yeast STKs also being found to be adenine-linked (Kaufman & Alivisatos, 1955; Schwartz *et al.*, 1983). These results lead to the assumption that all animal STKs were guanine-linked, whereas bacterial and plant STKs were adenine-linked. However, Hansford (1973) reported an adenine-linked STK from blowfly flight muscle and subsequently other workers (McClellan & Ottaway, 1980) demonstrated the wider occurence of adenine-linked STK in a range of animal tissues, though not mammalian. Indeed, Burnham (1963) demonstrated that the enzyme from the photosynthetic bacterium *Rhodopseudomonas spheroides* could utilize ADP, GDP or IDP almost equally well. Further investigation of the *E. coli* enzyme has revealed that GDP and IDP can serve as substrate, albeit less effectively than ADP (Murakami *et al.*, 1972). Several other bacterial

STKs have been examined for their nucleotide utilization and variation in ADP, GDP and IDP utilization has been detected (Kelly & Cha, 1977).

On the basis of these results and the molecular size diversity exhibited by STK, an examination was undertaken of the nucleotide substrate specificity of a range of bacterial STKs (Weitzman & Jaskowska-Hodges, 1982). This revealed a distinct pattern of specificity which showed correlation with bacterial classification (see Figure 5). The Km values for the nucleotides appear to present four distinct classes of bacterial STKs. Groups I, II and III all comprise Gram-negative bacteria, whereas Group IV are all Gram-positive bacteria. Group I bacteria resembling *Acinetobacter lwoffi* (aerobic organisms) possess STKs with a very high Km for ADP (~1 mM) but a much lower Km for GDP (~0·02 mM). Group II, of which *Pseudomonas aeruginosa* is a representative, exhibit similar and low Km values (~0·05 mM) for both ADP and GDP, while Group III, in which the enterobacteria occur, show low Km values for ADP (~0·01 mM) but significantly higher Km values for GDP (~0·1 mM). Group IV STKs appear only to function with ADP (Km values ~0·1 mM) displaying no activity with GDP as a substrate. It is worth noting that the same patterns of nucleotide substrate specificity are observed if the succinate thiokinase reaction is assayed in the reverse direction, in which the hydrolysis of ATP or GTP is coupled to the formation of succinyl-CoA.

Figure 5 Nucleotide specificity patterns of bacterial STKs

Group	Organism	K_m ADP	K_m GDP
I	*Acinetobacter anitratus* *Acinetobacter calcoaceticus* *Bordetella bronchiseptica* *Brevibacterium leucinophagum* *Chromobacterium violaceum* *Mima polymorpha* *Xanthomonas hyacinthi*	Very high	Low
II	*Paracoccus denitrificans* *Pseudomonas aeruginosa* *Pseudomonas fluorescens* *Pseudomonas stutzeri* *Rhodopseudomonas sphaeroides* *Thermus aquaticus*	Low	Low
III	*Alcaligenes faecalis* *Arizona arizonae* *Cellulomonas cellasea* *Escherichia coli* *Klebsiella (Aerobacter) aerogenes* *Serratia marcescens*	Low	High
IV	*Arthrobacter simplex* *Bacillus megaterium* *Bacillus stearothermophilus* *Brevibacterium linens* *Kurthia zopfii*	Low	No activity

Such diversity in nucleotide utilization and molecular size of various STKs poses intriguing questions as to their significance and participation in metabolic control and cellular processes.

1·4 Detailed Molecular Characterization of STK

1·4·1 Substrate specificity

Nucleotides & inorganic phosphate

Nucleotide substrate specificity has already been discussed (see Section 1·3). However, it is worth noting that the pig heart enzyme has shown the ability to utilize certain guanine analogues such as 8-aza GDP/GTP and 6-thio GTP, albeit with lower affinities (Cha, 1969). The *R. spheroides* enzyme, as previously mentioned, utilizes ATP, GTP and ITP equally effectively, but no activity was detectable with the pyrimidines CTP or UTP (Burnham, 1963). Replacement of inorganic phosphate with arsenate in the forward direction leads to arsenolysis of succinyl-CoA which is independent of nucleotide phosphorylation and occurs at a rate of about 20% that of the phosphorolysis system. Phosphorylation of the enzyme from *E. coli* and pig heart has been shown to occur in the presence of Pi + succinyl-CoA (Kreil & Boyer, 1964) or ATP and GTP respectively (Brownie & Bridger, 1972). It is now well established that the enzyme does indeed undergo phosphorylation during the catalytic reaction (see Section 1·4·4).

Succinate & CoA

Surprisingly, Wang and co-workers (Wang *et al.*, 1961) reported that itaconate could substitute for succinate with the pig heart enzyme although the affinity of the enzyme for itaconate was not reported. For the *E. coli* enzyme only malate, out of a possible eleven organic acids tested as possible substrates, was utilized (Robinson *et al.*, 1969). However, the activity with malate was 3% of that obtained with succinate and only observed at very high concentrations ($\geqslant$ 90 mM).

Only dephospho-CoA has been shown able to replace CoA in the catalytic reaction (Cha *et al.*, 1967).

Divalent metal ion requirement

There is an absolute requirement for a divalent metal ion in the succinate thiokinase reaction. The magnesium ion (Mg^{2+}) is the most effective and widely employed cation in all STK assays. After magnesium, in decreasing order of effectiveness, are manganese and cobalt ions, with only slight activity being observed with zinc and calcium ions (Gibson *et al.*, 1967). However, Mg^{2+} and Mn^{2+} have been reported to be equally effective at 6 mM with the pig kidney enzyme (Mazumder *et al.*, 1960). A divalent metal ion is probably required to complex with the nucleotide for correct orientation of the nucleotide in the active site. Evidence for a divalent metal ion binding site, at or near the active site region, has been

obtained using electron paramagnetic resonance (EPR) (Buttlaire *et al.*, 1977).

1·4·2 The *E. coli* Enzyme

Molecular weight & subunit organization

Studies involving subunit cross-linking combined with SDS-polyacrylamide gel electrophorensis have revealed the $\alpha_2\beta_2$ structure of the enzyme, with no evidence of cross-linked α_2, β_2 or $\alpha\beta$ pairings (Teherani & Nishimura, 1975). Molecular weight estimates for the tetramer, of between 136,000 - 146,000 (Krebs & Bridger, 1974; Ramaley *et al.*, 1967) with a Mr of 29,600 ± 500 for the α subunit and a Mr of 38,700 ± 300 for the β subunit (Bridger, 1971) have been shown to be in very close agreement with the Mr calculated from the primary structure of the enzyme (Buck *et al.*, 1986). The α and β subunits are derived from the *suc* D and *suc* C genes, respectively (Buck *et al.*, 1986); the Mr of the α subunit is 29,000 and that of the β subunit is 41.400. Significantly, the $\alpha_2\beta_2$ structure has now been confirmed as the active form of the enzyme by means of active enzyme centrifugation over a wide enzyme concentration range (Wolodko *et al.*, 1986).

Crystallization of the enzyme has been achieved (Wolodko *et al.*, 1984) as a prequisite for studying its 3-dimensional structure. Preliminary X-ray diffraction studies have suggested that the αβ dimers of the enzyme are arranged asymmetrically in the tetramer, as would be

expected if the alternating site co-operativity model is operational in the catalytic mechanism (see Section 1·4·4)

Subunit composition

The α and β subunits are not only distinct in their size but also in their amino acid composition. The recent determination of the primary structure (Buck *et al.*, 1985) shows very good agreement with the previous amino acid analysis of the isolated subunits (Bridger, 1974), with the subunit amino-terminal residues serine (α) and methionine (β) being confirmed. The α subunit is rich in glycine but deficient in asparagine, glutamate and serine and contains no tryptophan residues (Prasad *et al.*, 1983), whereas the β subunit, although high in glycine and low in serine, contains 3 tryptophan residues per mol. Between 16-18 titratable sulphydryl groups per enzyme molecule have been reported (Nishimura *et al* ., 1973). However, a total of 24 cystene residues occurs, 6 per subunit (Buck *et al.*, 1985). This means that either there are 6-8 inaccessible sulphydryls or that 3-4 disulphide bonds occur in the enzyme. For details concerning the essential amino acids in substrate binding and catalysis see Section 1·4·4.

1·4·3 The Pig Heart Enzyme

Molecular size & subunit organization

Molecular weight estimates for the enzyme range from

between 70,000 - 80,000 (Cha *et al.*, 1967; Murakami & Nishimura, 1974). The enzyme is reported to consist of only one α and one β subunit of Mr values, 34,500 and 42,300 respectively (Brownie & Bridger, 1974). Subsequent purification of the rat liver enzyme has yielded very similar molecular weights for the enzyme and subunits (Ball & Nishimura, 1980). Active enzyme centrifugation, as used to show the *E. coli* enzyme was a non-dissociating $\alpha_2\beta_2$ tetramer, has confirmed that the active form of the pig heart enzyme is a non-associating αβ dimer (Wolodko *et al.*, 1986).

Subunit composition

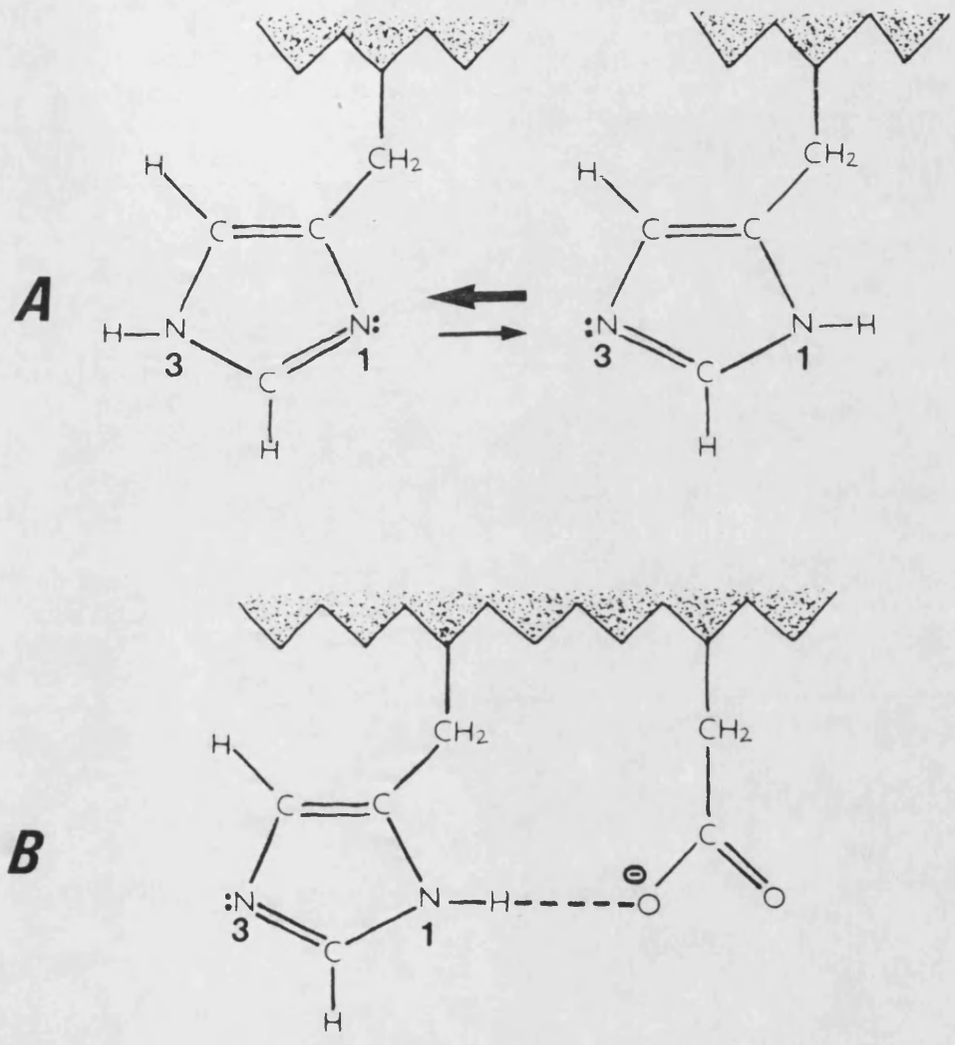
Far less work has been carried out on the pig heart enzyme, partly due to the low yields obtained by purification. However, amino acid analysis of the α and β subunits indicates, as with the *E. coli* enzyme, a lack of homology between the subunits. Interestingly, only one tryptophan residue has been found present on the β subunit (Nishimura, 1986), as compared with the 3 on each *E. coli* β subunit. The amino acid composition would predict 14 sulphydryl groups/mol of enzyme (Nishimura, 1986). However, only 12 sulphydryl groups are detectable (Murakami & Nishimura, 1974). Therefore, again, either a disulphide bond occurs within the native enzyme or 2 sulphydryl residues are inaccessible to the reagents used.

1·4·4 The Molecular Mechanisms of STK Substrate Binding Sites

In both the *E. coli* and pig heart enzymes a histidine residue located on the α subunit, identified in the *E. coli* enzyme as his-246 (Buck *et al.*, 1985), has been known for some time to be the residue phosphorylated by ATP/GTP in position N-3 of the imidazole ring (Kreil & Boyer, 1964; Vogel & Bridger, 1983) (see Figure 6). STK was the first enzyme demonstrated to involve a phosphohistidine in catalysis. Subsequent enzymes shown to utilize a phosphorylated histidine residue include ATP-citrate lyase, nucleotide diphosphokinase and the heat-stable bacterial phosphoryl-transfer protein (Nishimura & Grinnell, 1972).

The fact that isolated α subunits undergo autophosphorylation with ATP (Pearson & Bridger, 1975) indicated that this subunit contains the nucleotide binding site and the catalytic machinery for phosphoryl transfer. However, the rate of phosphorylation was greatly increased in the presence of the β subunit, indicating that the active site may also involve the β subunit. Significantly, phosphorylation of the α subunit by Pi and succinyl-CoA is only possibly in the presence of the β subunit (Kreil & Boyer, 1964). The concept that the nucleotide binding site is located at the α/β interface is further supported by affinity labelling studies. Using the analogues 2', 3'-dialdehyde-GDP for the rat

Figure 6 Tautomeric forms of the histidine imidazole ring



(A) The tautomeric form of the imidazole ring favoured in most proteins contains the lone pair of electrons on position (N-1) (Blomberg *et al.*, 1977).

(B) In STK phosphorylation occurs at position (N-3), it has been proposed that an anionic group stabilizes this tautomeric form (Vogels & Bridger, 1983).

liver enzyme (Ball & Nishimura, 1980) and 2', 3'-dialdehyde-ADP for the *E. coli* enzyme (Nishimura *et al.*, 1983), inactivation was shown to be accompanied by cross-linking of the α and β subunits.

The location of the succinate and CoA binding sites appears to be the β subunit. [^{14}C] succinate is known to bind covalently to the *E. coli* subunit, in the presence of ATP and CoA (Pearson & Bridger, 1975b) but, as yet, no studies on the residues involved have been presented. The CoA binding site in both *E. coli* and pig heart enzymes appears to contain at least one sulphydryl group on the β subunit (Collier & Nishimura, 1978). Inactivation by CoA affinity analogues that react with active site sulphydryl groups, was completely reversible with dithiothreitol.

Additional evidence that the active site is located at the α/β interface comes from studies involving the tryptophan residues, found exclusively on the β subunits. The maximum fluorescence emission of STK occurs at 335 nm. Acrylamide quenching of this fluorescence is significantly increased, in both enzymes, by the respective nucleoside triphosphate and by CoA. However, in contrast to the *E. coli* enzyme, the fluorescence of the pig heart enzyme appears to be protected from the acrylamide quenching by succinate (Prasad *et al.*, 1983; Nishimura, 1986).

Interestingly, chemical modification of a single tryptophan residue in isolated β subunits (*E. coli*) leads,

upon reconstitution with unmodified α subunits, to a refolded enzyme which, although inactive in the overall reaction, still has the ability to undergo autophosphorylation with ATP (Nishimura *et al.*, 1984).

It has now become widely accepted that the active site of STK is located at the point of contact between the α and β subunits. Substrate binding sites appear to be shared between the two subunits, being located very near the α/β interface.

The Reaction Mechanism

The specific steps of the STK reaction have for a long time been in dispute. Unlike the ATP-generating reactions of glycolysis, which effect phosphoryl transfer from an activated phosphorylated metabolite to ADP, the STK reaction utilizes inorganic phosphate with two other substrates and must therefore operate through a more complex mechanism.

The realization that the enzyme is phosphorylated during catalysis led to a number of proposed mechanisms, of which two gained credence:

Mechanism I

$$E + \text{succinyl-CoA} \rightleftharpoons E\text{-CoA} + \text{succinate}$$

$$E\text{-CoA} + Pi \rightleftharpoons E\text{-P} + CoA$$

$$E\text{-P} + NDP \rightleftharpoons E + NTP$$

Mechanism II

$$E + \text{succinyl-CoA} + Pi \rightleftharpoons E.\text{succinyl-P} + CoA$$

$$E.\text{succinyl-P} \rightleftharpoons E\text{-P} + \text{succinate}$$

$$E\text{-P} + NDP \rightleftharpoons E + NTP$$

From mechanism I, an enzyme-bound CoA intermediate would be predicted. However, ^{18}O studies revealed that the expected transfer of oxygen from the enzyme or CoA to inorganic phosphate did not occur and this was interpreted as evidence against an enzyme-bound CoA intermediate during catalysis (Benson *et al.*, 1964). Other ^{18}O exchange studies have demonstrated oxygen transfer between succinate and phosphate during the catalytic reaction (Cohn, 1959), thus giving support to mechanism II and the existence of an enzyme-bound succinyl-phosphate. Further direct evidence for such a covalent intermediate came from detection of its NMR signal (Vogel & Bridger, 1982) when ATP was added to a solution containing succinate and the phosphorylated enzyme. The possible reason for the ATP requirement in the formation of the succinyl-phosphate intermediate is discussed below.

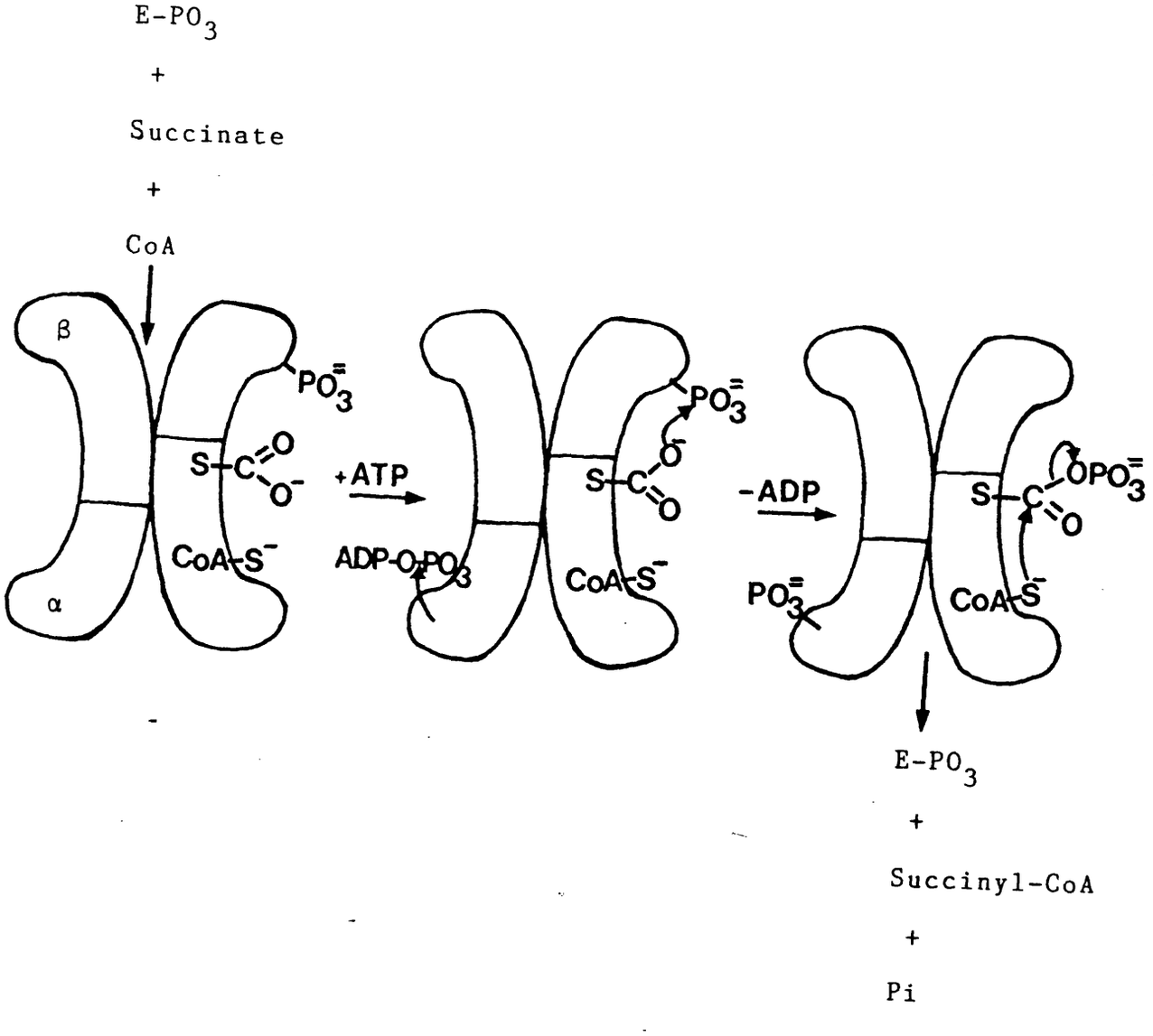
Half sites reactivity and Alternating site co-operativity

Half-sites reactivity (Moffet *et al.*, 1972) is the term given to observations that, although the tetrameric enzyme possesses two possible phosphorylation sites (his -α 246), only one phosphoryl group appears to be

incorporated per $\alpha_2\beta_2$ molecule. It was subsequently shown that both α subunits are equally available for phosphorylation (Wolodko *et al.*, 1981). Unfortunately, discrepancies exist concerning the number of phosphoryl groups binding to the tetramer, with ratios of 1·6 - 1·8 mol Pi/mol enzyme reported (Nishimura, 1986). These higher ratios are suggested to be produced by enzyme which is almost completely dephosphorylated, whereas the enzyme which is already phosphorylated would yield a ratio of one. However, the concept of half-site reactivity and other evidence has led to a conformation model for the enzyme during catalysis, known as alternating site co-operativity (Bild *et al.*, 1980). In this model the two αβ halves are viewed as being arranged asymmetrically to each other (see Figure 7). Evidence from preliminary X-ray diffraction studies appears to confirm such asymmetry in the enzyme (Wolodko *et al.*, 1984). It is proposed that the synthesis and release of succinyl-CoA occurring at one active site would be facilitated, due to structural rearrangements, by the binding of ATP at the second site. After undergoing phosphorylation by ATP the second site would in turn become the site for further succinyl-CoA synthesis, so that the continual turnover of the enzyme would occur in an alternating co-operative fashion between the two active sites.

This model has been tested by a number of methods. As previously mentioned, ^{31}P NMR studies (Vogel & Bridger,

Figure 7 Alternating site co-operativity model



1982) confirmed the existence of the enzyme-bound succinyl-phosphate intermediate. The requirement for ATP is consistent with the model, which indicates that the formation of succinyl-phosphate and ultimately succinyl-CoA, at one active site requires binding of ATP at the other site. The replacement of ATP with a non-hydrolysable ATP analogue demonstrated that phosphorylation at the second site was responsible for the co-operative effect (Vogel & Bridger, 1982). The model would also suggest that the rate of dephosphorylation of the enzyme by succinate and CoA should be stimulated by ATP, since the formation of succinyl-phosphate appears to be dependent upon the interaction of ATP with the neighbouring site. To study this turnover a thiophosphorylated tetrameric enzyme was prepared (Wolodko *et al.*, 1983). Stimulation of PSO_3^{3-} release, in the presence of succinate and CoA, occurred on addition of ATP exactly as predicted by the model. Alternating site co-operativity is believed to be a unique phenomenon dependent upon the subunit organization of the *E. coli* STK as an $\alpha_2\beta_2$ tetramer, so that the mammalian enzyme, which is a simple non-associating $\alpha\beta$ dimer (Wolodko *et al.*, 1986), would not be expected to display such stimulation. However, similar stimulation of PSO_3^{3-} release, by GTP, has been observed in the pig heart enzyme (Nishimura & Mitchell, 1985). This surprising result has been attributed either to a second binding site for GTP on

the enzyme or to a mechanism involving the incoming phosphoryl group of GTP displacing the phosphate already attached to the enzyme. It is therefore apparent that the precise molecular mechanisms involved in the STK reaction have yet to be finally established.

1·5 Synthesis of STK

The majority of researchers have concentrated their attention upon the mechanism of the STK reaction, so that little knowledge of the control of STK biosynthesis exists. Recent work in *E. coli* has identified the genes specifying the α and β subunits, locating them to the distal segment of a cluster of nine citric acid cycle enzymes (Buck *et al.*, 1985). Studies have also begun with the rat liver enzyme. Already the α subunit has been partly sequenced and established as having a cytoplasmic origin, complete with a cleavable signal sequence responsible for targetting its mitochondrial location (Bridger *et al.*, 1986).

1·6 Possible *in vivo* associations of STK

Multienzyme complexes appear to be present in most metabolic pathways. A number of studies have indicated specific interactions between various enzymes of the citric acid cycle, in both eukaryotic (Srere, 1985) and prokaryotic organisms (Barnes & Weitzman, 1986). Purified STK has been shown specifically to interact with purified

2-oxoglutarate dehydrogenase (Porpaczy *et al.*, 1983) and some loose association of STK with the inner mitochondrial membrane has been reported (Nishimura, 1986).

1·7 Possible physiological roles of STK

"Succinate thiokinase is one of the most neglected enzymes of intermediary metabolism" (Ottaway *et al.*, 1981). Such sentiment is well founded. STK, probably due to the freely reversible reaction it catalyses, has for a long time been regarded as a necessary but uninteresting link between the 2-oxoglutarate and succinate dehydrogenases. So much so that it is often excluded from flow diagrams of the cycle (Walsh *et al.*, 1986). As can be appreciated from Section 1·4, most devotees of STK have concentrated their efforts on understanding the molecular mechanisms of the reaction, rather than establishing any metabolic or physiological involvement.

Succinyl-CoA, besides its involvement in the citric acid cycle, is known to be required for the utilization of ketone bodies in extra-hepatic tissues. Ottaway and co-workers (Ottaway *et al.*, 1981) have suggested that only a guanine-linked STK (G-STK) and not an adenine-linked STK (A-STK) is found in tissues actively using ketone bodies. On the basis of computer stimulation studies and the predicted internal mitochondrial GTP/GDP ratios, they have predicted that, in such tissues, G-STK must be inhibited by the product GTP, before ketone body activation can

occur (McClellan & Ottaway, 1980). However, no experimental evidence has been presented to substantiate this proposal.

Another role for succinyl-CoA occurs is the biosynthetic pathway of porphyrins. This pathway is initiated by the condensation of succinyl-CoA and glycine by the enzyme δ-aminolevulinic acid synthase (ALA-S), to produce δ-aminolevulinic acid (ALA) (Shemin, 1979) which is then subsequently converted in several steps to haem. A number of genetically inherited diseases, known as "porphyrias", have been identified, in which dysfunction of an enzyme of the pathway leads to a compensatory increase in ALA-S activity (Elder, 1983a); porphyria may also be induced by a range of chemicals. Elevation of ALA-S may lead to a drain on succinyl-CoA and glycine. Most attention has focused on the regulation of ALA-S and subsequent enzymes, except for one report over 20 years ago (Labbé *et al.*, 1965) which describes the increase of STK activity in porphyric liver. This finding has largely been overlooked.

It is clear that, unlike the molecular aspects of STK, the possible metabolic associations of STK requires further investigation.

1·8 Aims and scope of thesis

After the establishment of distinct nucleotide specificity and molecular weight patterns among bacterial

STKs, it was decided that a careful study of STKs from eukaryotic organisms was necessary and timely. The interesting, if inconclusive, studies by Ottaway and co-workers (McClellan & Ottaway, 1980; Ottaway *et al.*, 1981) on the physiological significance of STK nucleotide specificity, together with the other metabolic roles of succinyl-CoA within mitochondria, have prompted further examination of various systems.

In this thesis, a broad range of organisms has been used to explore the diversity and physiology of the STK system. The studies encompass bacteria, eukaryotic unicellular organisms such as yeast and parasitic protozoa, plants, insects and mammals. The deliberate choice of these disparate organisms was designed to clarify the metabolic roles of STK. Studies have included comparative measurement of enzyme levels, enzyme fractionation and molecular and kinetic characterization.

Due to the wide diversity of organisms and metabolic conditions studied, the thesis is divided into separate chapters. Each chapter contains a short introduction to, and interpretations of, the results presented. The final chapter contains an integrated discussion of the discoveries that have been made and attempts to build them into a coherent understanding of the cellular roles of STK.

CHAPTER 2

MATERIALS & METHODS

CHAPTER 2

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MATERIALS

2·1 Organisms

The following bacterial strains were used: *Acinetobacter calcoaceticus* - 4B, *Arthrobacter simplex* - C73, *Bacillus megaterium* - D101, *Bacillus subtilis* - 168, *Brevibacterium linens*, *Brochothrix thermosphacta*, *Corynebacterium fascians*, *Corynebacterium rubrum*, *Deinococcus radiodurans*, *Escherichia coli* - K12D500, *Kurthia zopfii* and *Staphylcoccus aureus* - 12600. All strains were obtained from the culture collection of Prof. P.D.J. Weitzman, University of Bath.

Saccharomyces cerevisiae (Baker's yeast) from the Microbiology Group, University of Bath.

Trypanosoma brucei bloodstream strain - MITat1·1 and procyclic strain - EATRO - 427, were originally obtained from Dr H.P. Voorheis, Trinity College, Dublin and Dr W. Gibson, Tsetse fly centre, Langford, Bristol, respectively, being subsequently prepared and kindly supplied by Dr R. Eisenthal, University of Bath.

Mouse lymphoma - L1210 and hamster kidney (BHK) cell lines were obtained from Dr W.J.D Whish, University of Bath.

Locusta schistocerca gregaria (desert locust) were obtained from Dr G.G. Lunt, University of Bath.

Wistar rats and white C.F.L.P.-mice were obtained from the maintained stocks at the University of Bath.

Other animal tissues, eg. bovine, porcine and ovine, were obtained from a local abattoir after slaughter.

Zea maize (jubilee), etiolated and non-etiolated, were obtained from the Horticulture Group, University of Bath. Pigeon (racing) breast muscle was obtained from University of Bristol.

2:2 Chemicals & Enzymes

All inorganic chemicals were obtained at highest purity from BDH Chemicals Ltd., Poole, Dorset, U.K. and Fisons Scientific Apparatus, Loughborough, Leics., CoA, OAA, NADP and NADPH were from Boehringer Mannheim, Lewes, Sussex, U.K., adenosine, guanosine and inosine nucleotides, acetoacetyl-CoA, bovine thyroglobulin, lithium acetoacetate, mesoporphyrin, sodium β-hydroxybutyrate, streptozotocin, succinic anhydride and all other fine chemicals were from Sigma Chemical Co., Poole, Dorset, U.K.

Nutrient agar and broth were from Oxoid Ltd., London, U.K., Clinistix and Ketostix came from Ames Division, Miles Laboratories Ltd. (supplied through Arnold R. Harwell Ltd., London). Insulin (Neulente-zinc suspension, 100 I.U./ml, Wellcome) was kindly supplied by Dr J.P.D. Reckless, Royal United Hospital, Bath. Bio-Rad

protein assay solution and hydroxylapatite (Bio-gel HTP) were obtained from Bio-Rad Laboratories Ltd., Watford, U.K., Percoll, PD-10 columns, Sephadex G-10, Sephacryl S-200 and Sepharose 4-B were from Pharmacia, Milton Keynes, U.K., Zorbex-ODS (4·6 x 250 mm) and Zorbex-NH_2 (4·6 x 250) HPLC columns (Du Pont) were obtained through Anachem, Luton, U.K.. The triazine dyes were kindly supplied by Dr S. Syed. 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) was kindly supplied by Dr F. De Matteis, MRC Toxicology Unit, Carshalton.

Enzymes

Glucose 6-P dehydrogenase (yeast), lactate dehydrogenase (rabbit muscle), malate dehydrogenase (pig heart), phosphoglycerate kinase (yeast) were from Boehringer Mannheim. β-hydroxybutyrate dehydrogenase (*Rhodopseudomonas spheroides*) and succinate thiokinase (pig heart) were obtained from Sigma Chemical Co.

2·3 Polarography

Due to the unusual use of this electroanalytical technique for the assay of enzymic activity, a short introduction to the subject and the practical procedures involved has been included.

2·3·1 General principles & theory

Introduction

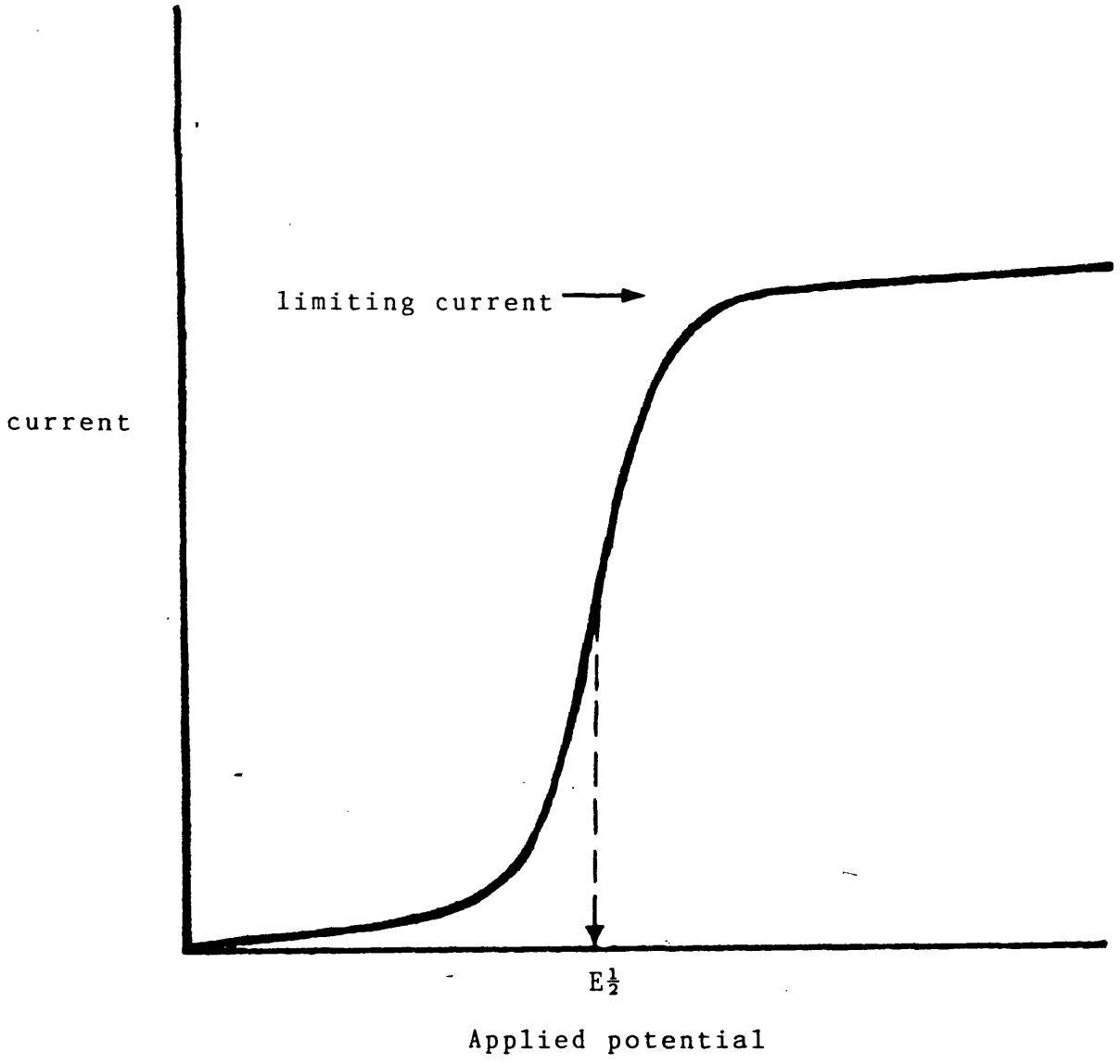
Polarography is an electrochemical method of

analysis, devised by Heyrovsky (1922). It involves the production of a current at a polarizable micro-electrode, as a function of the applied potential. The polarizable micro-electrode, usually a dropping mercury electrode, is immersed in the test solution, while a non-polarizable reference electrode, connected via a salt bridge to the test solution, completes the circuit. Reduction or oxidation of an electroactive species, at the microelectrode, produces a cathodic or anodic current, respectively.

The relationship between current and voltage is shown in Figure 8. As the potential difference is increased from zero, very little current will flow until the potential is such that the electroactive species can be reduced or oxidized at the surface of the micro-electrode. This causes a large increase in current from a relatively small increase in potential. The mid-point ($E\frac{1}{2}$) of the wave is characteristic of the particular species examined. As the potential difference is further increased, a situation is reached where the electroactive species is reduced or oxidized as fast as it reaches the microelectrode. The current is then said to be the "limiting current" and is determined by the rate of diffusion of the electroactive species, which is directly proportional to its concentration. Therefore the activity of enzymes that utilize or produce an electroactive substrate or product can be measured in a

Figure 8 Half-wave potential of an electroactive species

(relationship between current and applied potential)



continuous manner.

Considerations of current & diffusion

Any electrolytic process involves first the bringing of the electroactive species to the electrode surface and secondly the electrochemical process. The first step is associated with the diffusion of the electroactive species to the electrode, and therefore is temperature and concentration dependent. The electrochemical step, involving electron transfer between the electrode and species in solution, often requires adsorption followed by atomic or molecular rearrangements of the electroactive species into a form suitable for electron exchange. These rearrangements may be rapid or slow and therefore could be rate limiting. However, polarography has traditionally been concerned with rapid electrochemical processes, such that the rate-limiting step in the whole procedure is the diffusion of the electroactive species to the electrode.

With the dropping mercury electrode (d.m.e.) a near spherical drop of mercury, which slowly forms and then falls from the end of a very fine capillary, acts as the polarizable electrode. A sophisticated mathematical solution was required to solve the problem of quantifying the current produced, by the diffusion of an electroactive species to a spherical drop which is increasing in volume uniformly with time. This was

achieved by Ilkovic (1934).

Ilkovic equation:

$$i = 607\ n\ D^{\frac{1}{2}}\ C \cdot M^{\frac{2}{3}}\ t^{\frac{1}{6}}$$

(A) (B)

where i = current (μA)

607 = a numerical constant

n = number of electrons transfered per mol electroactive species

D = diffusion coefficient (cm^2/s)

C = concentration (mM)

M = mass of mercury flowing (mg/s)

t = formation time of one mercury drop

From the equation it is apparent that section (A) is dependent upon the solution, whereas section (B) is dependent upon the electrode. Therefore if the electrode and nature of solution are constant, the current is proportional to concentration.

2·3·2 The d.m.e., reaction vessel and reference electrode

A d.m.e. consists of a reservoir of pure mercury, of variable height, from which mercury escapes via a fine glass capillary (20-30μm diameter). The mercury issues from the capillary in regular and identical drops at a rate dependent upon the height of the reservoir (required drop time = 4-9S). The reaction vessel consists of a

glass cell, suitable for a 1 ml test solution, with a standard ground glass neck. This is attached to a matching stopper of hard polythene, which is held in a fixed vertical position and ensures a gas-tight seal for the system. Through the stopper are a number of drilled holes to carry: the d.m.e. capillary, a salt bridge (20% agar in saturated KCl) and inlet and outlet tubes for nitrogen. A small capped hole also exists to allow introduction of extract solution (see Figure 9). The applied potential is measured against a saturated calomel non-polarizable electrode. This reference electrode consists of a paste mixture of mercury (Hg) and mercurous chloride (Hg_2Cl_2) in a saturated KCl solution, so that an equilibrium is established:

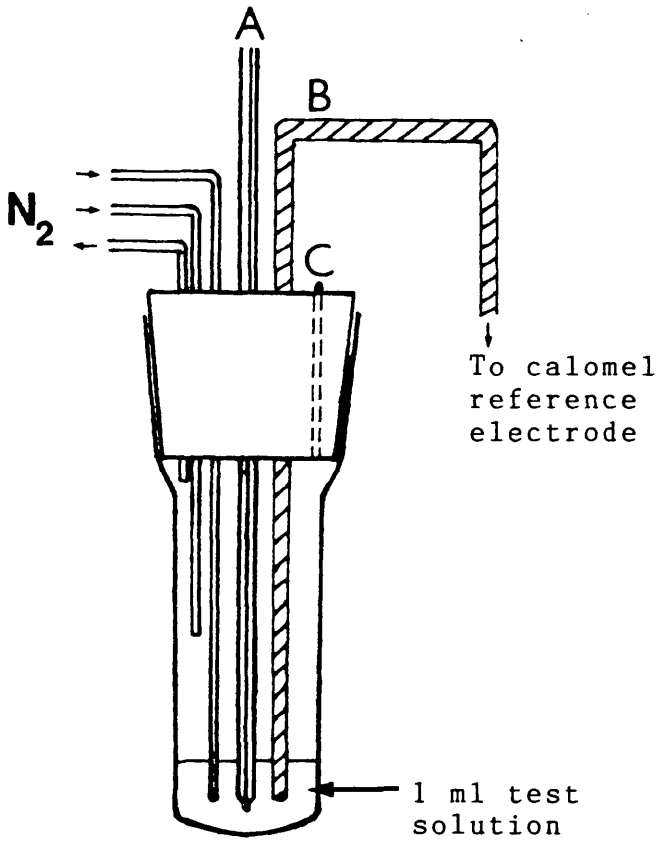
$$Hg_2Cl_2 + 2e^- \rightleftharpoons Hg + 2\ Cl^-$$

2·3·3 The CoA assay procedure

Enzymes responsible for the hydrolysis of acyl-CoA derivatives produce free CoA-SH which can be measured readily at a potential of -0·3 V (Weitzman, 1976). CoA-SH, but not its acyl-CoA derivatives, produces an anodic polarographic wave, as the CoA-SH is oxidised forming a mercaptan with the mercury:

$$Hg + 2\ CoA\text{-}SH \rightleftharpoons Hg(CoA)_2 + 2\ H^+ + 2\ e^-$$

Figure 9 Reaction vessel for polarography



A dropping mercury electrode

B salt bridge (2% agar in saturated KCl)

C small capped hole (to enable material to be added to vessel)

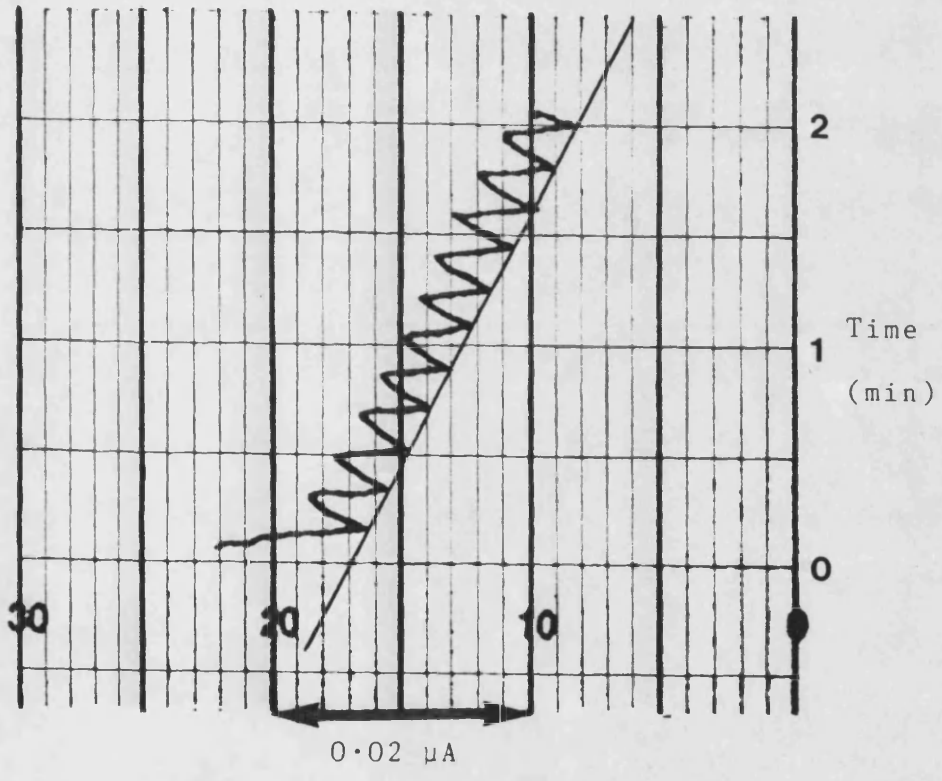
The assay mixture is placed in the reaction vessel which is then attached to the polythene stopper, so that the d.m.e., salt bridge and nitrogen inlet tube all reach into the solution. Oxygen-free nitrogen is then flushed through the solution for 2 min to remove oxygen from the system. This is necessary because oxygen has two redox potentials at + 0·8 and + 0·3 V (Morris, 1974), so that throughout the working potential of the d.m.e. molecular oxygen is reducible and would thus produce a polarographic wave that would mask other polarograms. After oxygen removal, extract is introduced into the assay mixture using a very fine pipette. The nitrogen tap is then quickly adjusted to allow nitrogen to flow over the surface of the solution so that turbulence ceases. Immediately a fixed potential is applied and the current recorded automatically with a Radiometer PO4 recording polarograph.

The trace obtained has a characteristic oscillatory pattern (see Figure 10). The advantage of using a d.m.e. over other types of electrode is that each drop that forms is unaffected by the reaction which occurred at the surface of the earlier drops. This ensures that the d.m.e. is never poisoned because the surface is continually renewed.

2·3·4 Calibration of the d.m.e.

As can be deduced from the Ilkovic equation, each mercury electrode requires calibration for a particular

Figure 10 Typical polarographic trace obtained from the d.m.e.



electroactive species. This is achieved by preparing a 1 mM solution of CoA-SH, the exact free thiol concentration being determined using DTNB (ε_{412} = 13·6 x 10^3 $l.mol^{-1}.cm^{-1}$). Known amounts of CoA are added to a solution of 0·1 M sodium/potassium phosphate buffer, pH 8·0, containing 10 mM $MgCl_2$ to give a final volume of 1 ml. A potential of -0·3 V is applied to each sample and the height of the anodic wave produced is recorded. A simple linear calibration plot is then constructed. Once calibrated the electrode is used at a fixed mercury column height. The electrode used for all enzyme assays had a conversion factor of 0·613 so that $\mu A\ ml^{-1}min^{-1}$ x 0·613 = $\mu moles\ ml^{-1}min^{-1}$.

2·3·5 Care of the d.m.e.

The use of pure, double or triple distilled mercury is essential for the uniform formation of each mercury drop. As each drop falls, the mercury thread momentarily retracts slightly into the capillary lumen before the succeeding drop begins to form. This pumping of solution in and out of the lumen, can over time foul the capillary and lead to erratic and slower mercury flow. Periodic immersion of the electrode, with the mercury flowing, into 50% (v/v) nitric acid, acetone and finally double distilled water will maintain a clean lumen.

2·3·6 Advantages & general applications

Polarographic measurement of STK activity is a particularly suitable assay procedure for examining nucleotide specificity and dependence of the enzyme. Other assays that have been employed are the hydroxamate method (Kaufman, 1955) which is discontinuous and rather insensitive (Weitzman & Kinghorn, 1980) and the spectrophotometric method at 235 nm (Cha, 1969). This continuous assay suffers from interference absorbance of the nucleotide substrates and is unsuitable for crude cell extracts of high turbidity. Similarly, measurement of STK activity by free CoA-SH detection, with the reagent DTNB, as used for citrate synthase assays (Srere *et al.*, 1963), may also be unsuitable. This method, although widely used is known to expose enzymes to the possibility of inactivation by the chromogen (DTNB) (Weitzman & Danson, 1976) and this is especially so with eukaryotic STK enzymes. However, all these possible disadvantages in traditional assay methods are eliminated by use of the polarographic procedure.

Many enzyme reactions involve substrates or products which are electroactive. Besides CoA-SH and other sulphur-containing compounds, recently reviewed by Adler & Westely (1987), the carbonyl group of pyruvate produces a cathodic wave at -1·4 V so allowing pyruvate kinase to be assayed. Lipoamide dehydrogenase activity may be measured by detection of either the cathodic or anodic waves produced by lipoamide and dihydrolipoamide respectively (at -0·75 V and -0·2 V), while NAD(P)-linked

dehydrogenase reactions may be assayed by following the reduction of NAD^+ or $NADP^+$, to produce a cathodic wave at -1·1 V (Weitzman, 1976).

In conclusion, the polarographic method of enzyme assay is a technique which has a wide and useful application in a variety of enzyme systems. It has been found to be particularly applicable and advantageous in the measurement of STK activities.

2·4 Enzyme assays

Polarographic assays were carried out using a Radiometer PO4 recording polarograph. Spectrophotometric assays were carried out with either a Perkin Elmer 504 or a Pye Unicam SP1800 spectrophotometer.

All assays were carried out at 25°C. A unit of enzyme activity is defined as that amount of enzyme which catalyses the conversion of 1 µmole of substrate to product per min. Where appropriate, non-specific reactions were taken into account. All assays involving the measurement of NAD/NADH/NADP/NADPH were calculated using $\varepsilon_{340} = 6{\cdot}22 \times 10^3$ $1.mol^{-1}.cm^{-1}$. Results are expressed in activity min^{-1} (mg of protein)$^{-1}$ or in activity min^{-1} (ml of extract)$^{-1}$.

Acetoacetyl-CoA thiolase [EC 2·3·1·9]

ACT was assayed by following the decrease in absorbance at 303 nm due to the hydrolysis of acetoacetyl-CoA ($\varepsilon_{303} = 20{\cdot}5 \times 10^3 \times 1.mol^{-1}.cm^{-1}$). The assay mixture contained 0·1 mM acetoacetyl-CoA, 0·11 mM CoA and 50 mM

$MgCl_2$ in 50 mM Tris-HCl buffer, pH 7·5. The reaction was initiated by the addition of the CoA.

Citrate synthase [EC 4.1.3.7]

CS was assayed by the method of Srere *et al.* (1963) using Ellman's reagent 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB), which is cleaved by CoA-SH. The increase in absorbance at 412 nm, due to the production of the yellow thio-nitrobenzoate anion, was followed spectrophotometrically (ε_{412} = 13·6 x 10^3 $l.mol^{-1}.cm^{-1}$). The assay mixture contained 0·2 mM acetyl-CoA, 0·2 mM OAA and 0·1 mM DTNB in 20 mM Tris-HCl + 1 mM EDTA buffer, pH 8·0. CS was also measured polarographically (Weitzman, 1969) with the same assay mixture except for the omission of DTNB. The formation of CoA-SH was monitored with a dropping mercury electrode at a potential of -0·3 V relative to a saturated calomel electrode, with a full scale deflection of 0·2 µA (µmoles $ml^{-1}min^{-1}$ = 0·613 x µA $ml^{-1}min^{-1}$).

Glutamate dehydrogenase [EC 1.4.1.3]

GDH was assayed by following the oxidation of NADH at 340 nm. The assay mixture contained 3 mM 2-oxoglutarate, 25 mM NH_4Cl and 50 µM NADH in 0·1 M sodium/potassium phosphate + 2 mM EDTA buffer, pH 7·5.

Hexokinase [EC 2.7.1.1]

HK was assayed by linking to glucose 6-P

dehydrogenase (G6PDH) and following the reduction of $NADP^+$ at 340 nm. The assay mixture contained 222 mM glucose, 8 mM $MgCl_2$, 0·91 mM $NADP^+$, 0·64 mM ATP and 0·155 U/ml G6PDH in 40 mM TEA buffer, pH 7·6.

β-Hydroxybutyrate dehydrogenase [EC 1·1·1·30]

HBDH was assayed by following the oxidation of NADH at 340 nm. The assay mixture contained 100 µM lithium acetoacetate and 0·2 mM NADH in 50 mM sodium/potassium phosphate buffer, pH 6·8.

Isocitrate dehydrogenase [EC_{NAD} 1·1·1·41] [EC_{NADP} 1·1·1·42]

IDH was assayed by following the increase in absorbance at 340 nm. The assay mixture contained 4 mM isocitrate, 0·2 mM NAD^+ or $NADP^+$ in MET-8 buffer. 1 mM ADP was included in the assay for the NAD-linked enzyme.

Lactate dehydrogenase [EC 1·1·1·27]

LDH was assayed by following the oxidation of NADH, as a decrease in absorbance at 340 nm. The assay mixture contained 0·2 mM pyruvate and 0·2 mM NADH in 20 mM Tris-HCl buffer, pH 8·0.

Malate dehydrogenase [EC 1.1.1.37]

MDH was assayed by following the decrease in absorbance at 340 nm. The assay mixture contained 0·2 mM OAA and 0·2 mM NADH in MET-8 buffer.

3-Oxo-acid CoA-transferase [EC 2.8.3.5]

OAT was assayed by following the decrease in absorbance at 303 nm due to the cleavage of acetoacetyl-CoA. (ε_{303} = 20·5 x 10^3 $l.mol^{-1}.cm^{-1}$). The assay mixture contained 80 µM acetoacetyl-CoA, 10 mM iodoacetamide, 10 mM $MgCl_2$ and 50 mM succinate in 50 mM Tris-HCl buffer, pH 8·5. The reaction was initiated by the addition of the succinate.

2-Oxoglutarate dehydrogenase [EC 1·2·4·2]

OGDH was assayed by following the increase in absorbance at 340 nm due to the reduction of NAD^+. The assay mixture contained 2 mM 2-oxoglutarate, 2·5 mM NAD^+, 0·2 mM TPP, 0·13 mM CoA, 2·6 mM cysteine-HCl and 1 mM $MgCl_2$ in 50 mM potassium phosphate buffer, pH 8·0.

Phosphoenolpyruvate carboxykinase (EC 4.1.1.32]

PEPCK was assayed by linking to MDH and following the decrease in absorbance at 340 nm, due to the oxidation of NADH. The assay mixture contained 1·25 mM phosphoenolpyruvate, 1·25 mM GDP, 50 mM $KHCO_3$, 1 mM $MnCl_2$, 0·15 mM NADH and 5 U/ml MDH in 100 mM imidazole-HCl + 2 mM DTT buffer, pH 6·6.

Phosphoglucose isomerase [EC 5.3.1.9]

PGI was assayed by linking to G6P-DH. The reduction of $NADP^+$ was followed at 340 nm. The assay mixture contained 1·4 mM fructose-6-phosphate, 6·8 mM $MgCl_2$,

0·39 mM $NADP^+$ and 0·46 U/ml G6P-DH in 85 mM TEA buffer, pH 7·6.

3-Phosphoglyceraldehyde dehydrogenase-$NADP^+$ [EC 1.2.1.9]

PGADH was assayed by following the oxidation of NADPH at 340 nm. The substrate 3-phosphoglycerate phosphate was produced in the assay from phosphoglycerate kinase. The assay mixture contained 1 mM 3-phosphoglycerate, 3·3 mM ATP, 1·5 U/ml phosphoglycerate kinase, 10 mM $MgCl_2$, 4 mM EDTA, 0·14 mM NADPH and 1 mM DTT in 67 mM MOPS buffer, pH 7·3. The assay mixture was incubated at room temperature for 1 min to allow activation, before initiation of the reaction by addition of the 3-phosphoglycerate.

Pyruvate kinase [EC 2.7.1.40]

PK was assayed by linking to LDH. The oxidation of NADH was followed at 340 nm. The assay mixture contained 0·54 mM phosphoenolpyruvate, 2·5 mM $MgSO_4$, 10 mM KCl, 4·7 mM ADP, 0·2 mM NADH and 9·2 U/ml LDH in 20 mM Tris-HCl buffer, pH 8·0. The reaction was initiated by the addition of the phosphoenolpyruvate.

Succinate thiokinase ADP [EC 6.2.1.5] GDP [EC 6.2.1.6]

STK was assayed polarographically in the forward direction (succinyl-CoA breakdown). CoA-SH formation was monitored with a dropping mercury electrode at a potential of -0·3 V, relative to a saturated calomel

electrode, with a full scale deflection of 0·2 μA (μmoles = 0·613 x μA). The assay mixture contained 0·17 mM succinyl-CoA, 0·5 mM NDP and 10 mM $MgCl_2$ in 100 mM sodium/potassium phosphate buffer, pH 8·0.

STK was measured spectrophotometrically in the reverse direction (succinyl-CoA formation) by following the increase in absorbance at 235 nm due to the thio-ester bond of succinyl-CoA (ε_{235} = 4·5 x 10^3 $1.mol^{-1}cm^{-1}$). The assay mixture contained 10 mM sodium succinate, 10 mM $MgCl_2$, 0·4 mM NTP and 0·17mM CoA in 50 mM Tris-HCl buffer, pH 7·5.

2·5 Estimation of Protein

Protein concentrations were estimated by the method of Bradford (1976). To 800 μl of test solution were added 200 μl of Bio-Rad reagent; after gently mixing the absorbance was read at 595 nm after 5 min. Bovine thyroglobulin was used as standard.

2·6 Preparation of reagents

2·6·1 Preparation of acetyl-CoA

Acetyl-CoA was prepared by the method of Stadtman (1957). To 10 mg of CoA, dissolved in 1 ml of double-distilled H_2O, was added 0·2 ml of 1M$KHCO_3$ to ensure a neutral pH. 0·2 ml of 0·1 M acetic anhydride was then added and the mixture incubated for 10 min on ice. Completion of acetylation was checked using DTNB.

2·6·2 Preparation of succinyl-CoA

Succinyl-CoA was prepared by the method of Simon & Shemin (1953). 5 mg of CoA were dissolved in 0·5 ml of double-distilled H_2O. A neutral pH was ensured by addition of 100 µl of 1M$KHCO_3$. 20 mg of succinic anhydride were added and the solution frequently and thoroughly mixed for 5 min. The excess succinic anhydride was then removed by filtration and the succinyl-CoA solution stored on ice.

2·6·3 Stability & estimation of succinyl-CoA

All acyl-CoA derivatives are notoriously unstable, with succinyl-CoA having a half-life of between 1-2 h at room temperature (Dawson *et al.*, 1986). The stability of succinyl-CoA, stored on ice, was therefore determined over a period of 24 h. Hydroxylamine is known to react with acyl-CoA derivatives to yield an acyl-hydroxamate and CoA; DTNB can then be added to quantify the CoA liberated.

To the succinyl-CoA sample, in 100 mM sodium/potassium phosphate buffer, pH 7·5, was added 50 µl of a 2·4 M neutralized hydroxylamine stock solution (3 ml of 4 M NH_2OH-HCl + 2 ml of 7 M KOH). After 15 min, 20 µl of 10 mM DTNB was added and the absorbance read at 412 nm (ε_{412} = 13·6 x 10^3 $1.mol^{-1}cm^{-1}$). The free CoA-SH content was determined by addition of DTNB to succinyl-CoA samples in the absence of hydroxylamine.

CoA (grade 1) obtained from Boehringer Mannheim is reported to be 85% pure. Therefore the maximum amount of succinyl-CoA possible from 5 mg CoA/600 µl would be 9 mM (Mwt CoA = 785·4). The concentration of succinyl-CoA measured by the hydroxylamine/DTNB method was found to be 8·7 mM, which indicates 97% succinylation of the original CoA. The stability of succinyl-CoA stored on ice was determined (see Table 1). Even after 5½ h only 12% of succinyl-CoA had been hydrolysed, as determined by the hydroxylamine/DTNB method. During the succinylation of CoA, succinic anhydride may also react with water to produce succinate.

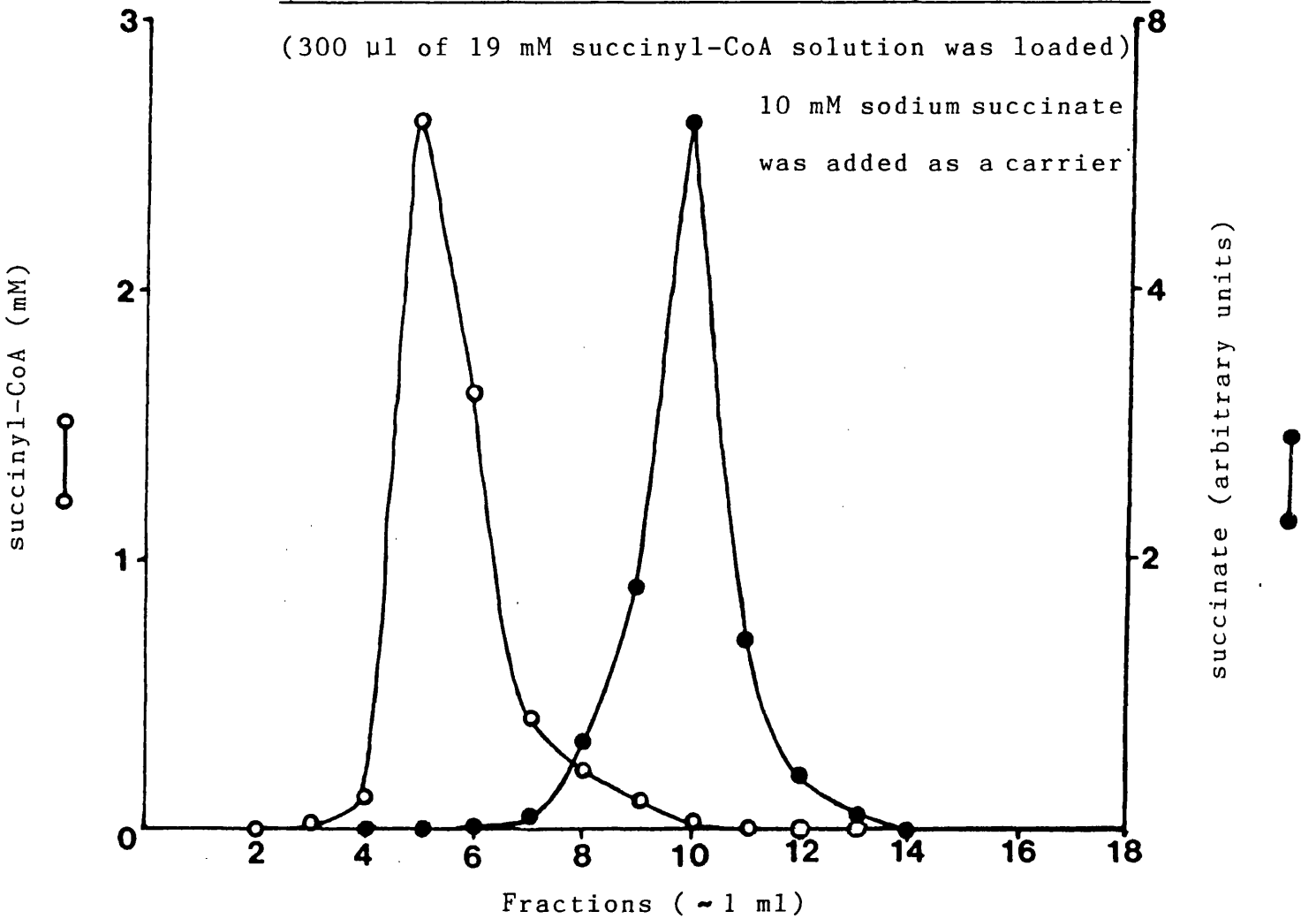
As succinate is a product of the STK reaction, separation of succinyl-CoA from succinate contamination was required to ensure that product inhibition did not produce interference. This was achieved on a column of Sephadex G-10 (2·5 x 400 mm) (see Figure 11).

Table 1 Stability of succinyl-CoA

Time (h : min)	- hydroxylamine (A_{412})	+ hydroxylamine (A_{412})	succinyl-CoA (mM)
0	0·01	1·2	8·75
1 : 50	0·013	1·2	8·7
2 : 40	0·077	1·2	8·25
5 : 30	0·15	1·2	7·7
*24 : 00	0·2	1·2	7·35

* Stored overnight at -21°C

Figure 11 Separation of succinyl-CoA from succinate on Sephadex G-10 (2·5 x 400 mm) in 50 mM MOPS buffer, pH 6·5, at 4°C

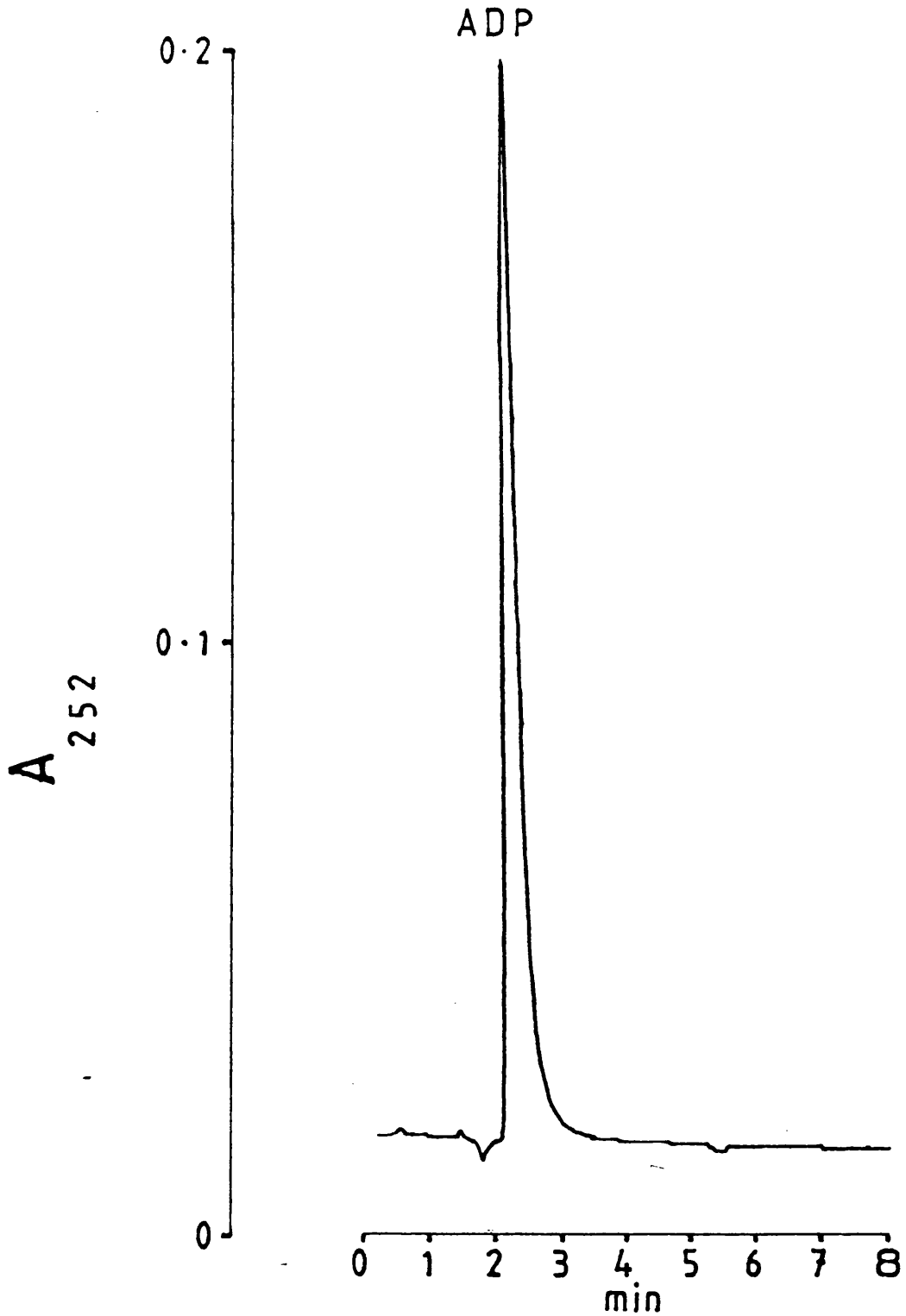


Succinyl-CoA was measured using the hydroxylamine/DTNB assay, whereas succinate was detected by using the reverse directional STK assay, by following the increased absorbance, due to succinyl-CoA formation, at 235 nm. This partially purified succinyl-CoA was shown to produce no significant increase in STK activities. However, for all kinetic studies partially purified succinyl-CoA was used. No such potential problem exists with the citrate synthase assay, where the acetyl-CoA also contains acetate, because acetate is not a product of the reaction.

2·7 Determination of the purity of adenosine, guanosine and inosine dinucleotides by HPLC

ADP, GDP and IDP were obtained from Sigma at the highest purity. To remove any possibility of there being cross-contamination between the nucleotides, HPLC analysis was carried out on a LDC/Milton Roy HPLC system. Initial studies using a Zorbex-ODS column (4·6 x 250 mm), with an isocratic elution of 10 mM sodium/potassium phosphate buffer, pH 6·5, appeared to confirm the individual purities (ADP = 94·7%, GDP = 96% & IDP = 97%) but failed to separate a mixture of all three nucleotides. Separation was achieved using a Zorbex-NH_2 column (4·6 x 250 mm) with an isocratic elution of 1·4% acetic acid, 2·8% methanol, 1% acetonitrile and 70 mM NaCl, pH 2·5 (see Figures 12, 13, 14 & 15). These results conclusively demonstrated the purity of the nucleotides used, and eliminate the possibility of erroneous STK

Figure 12 HPLC analysis of ADP on Zorbex-NH_2 column



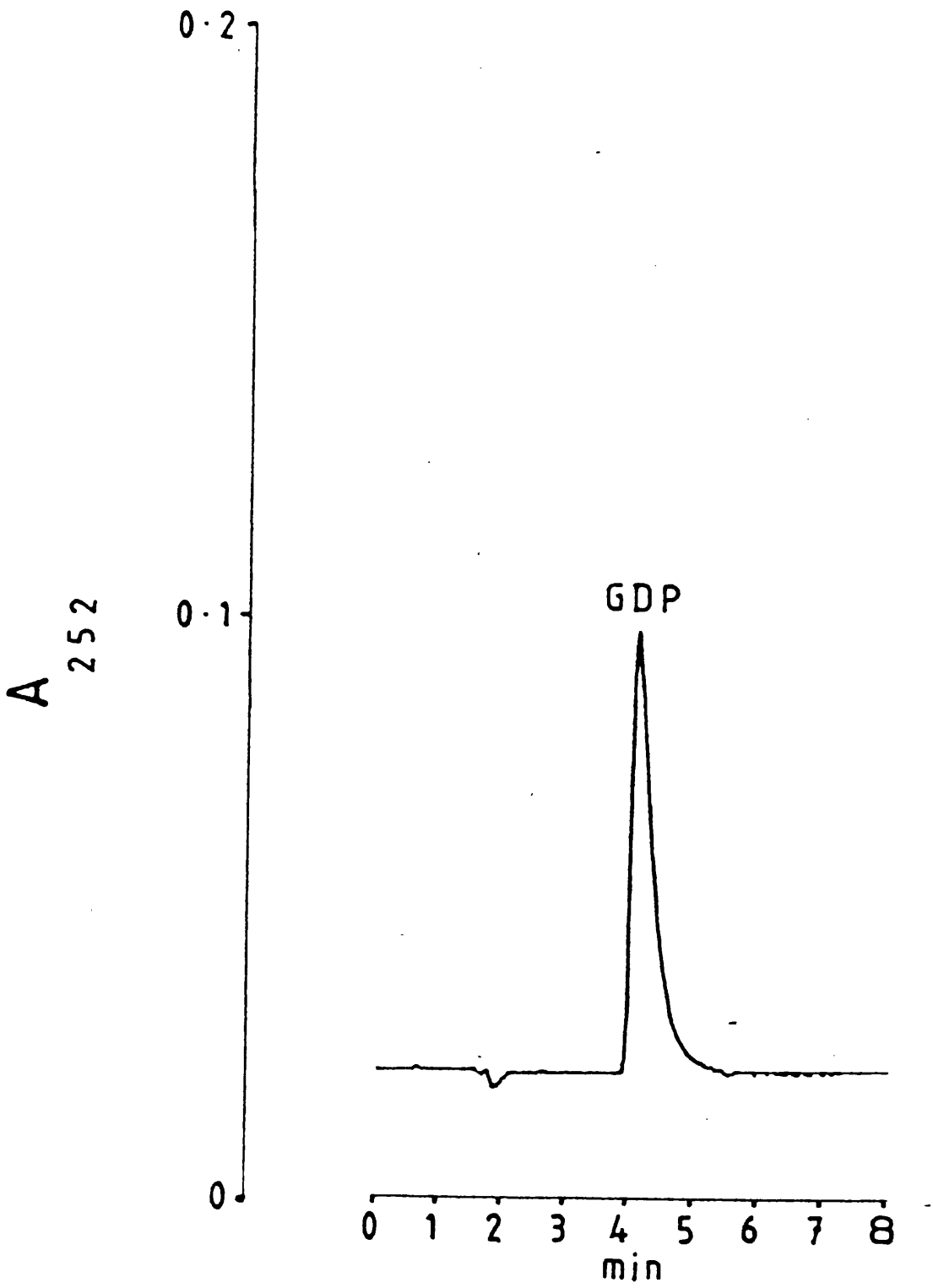
sample = 10 µl (10mM) ADP

flow rate = 2 ml/min

scale = 0-2·0

chart recorder = 10 mm/min

Figure 13 HPLC analysis of GDP on Zorbex-NH_2 column



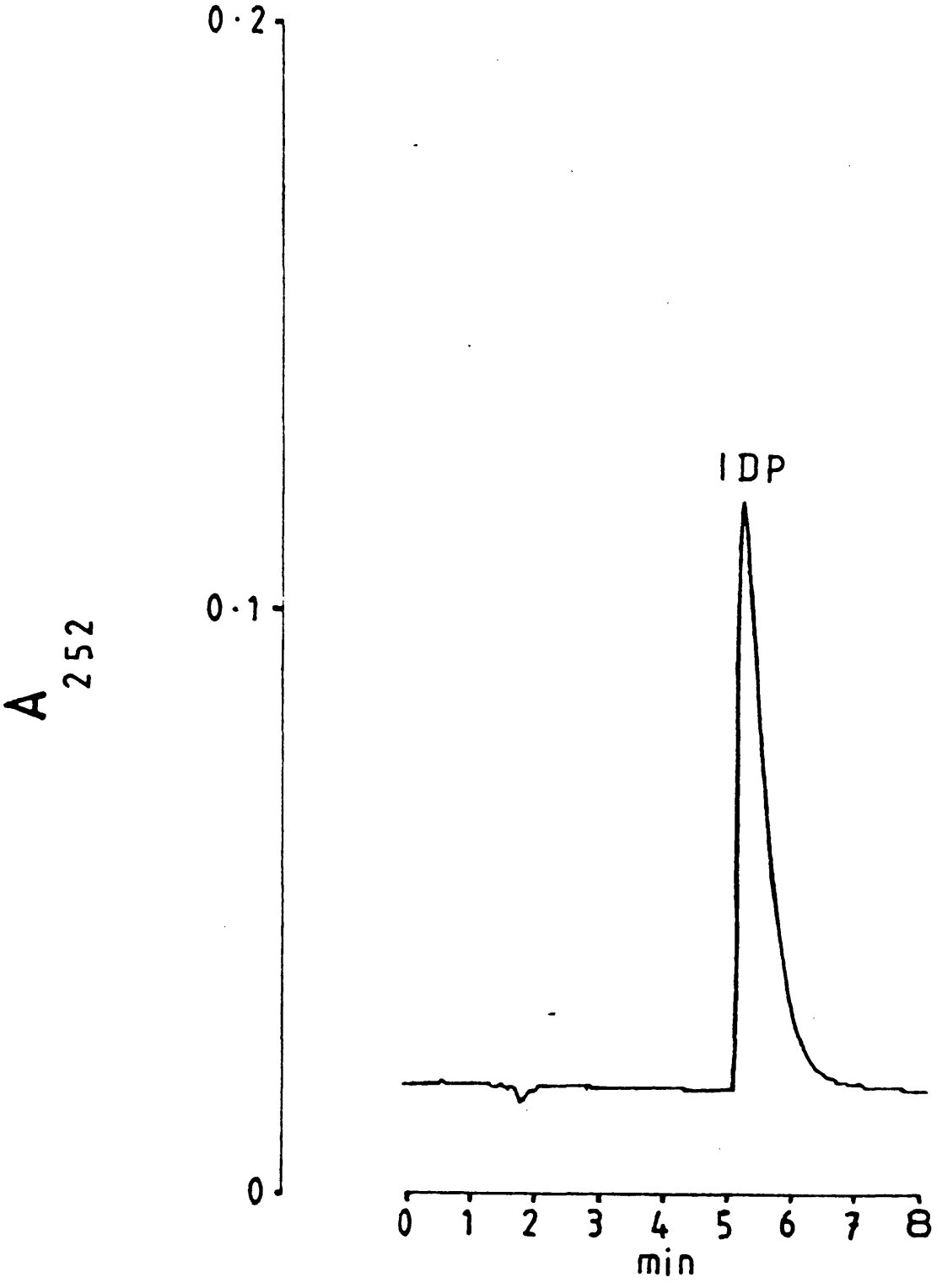
sample = 20 µl (10 mM) GDP

flow rate = 2 ml/min

scale = 0-2·0

chart recorder = 10 mm/min

Figure 14 HPLC analysis of IDP on Zorbex-NH_2 column



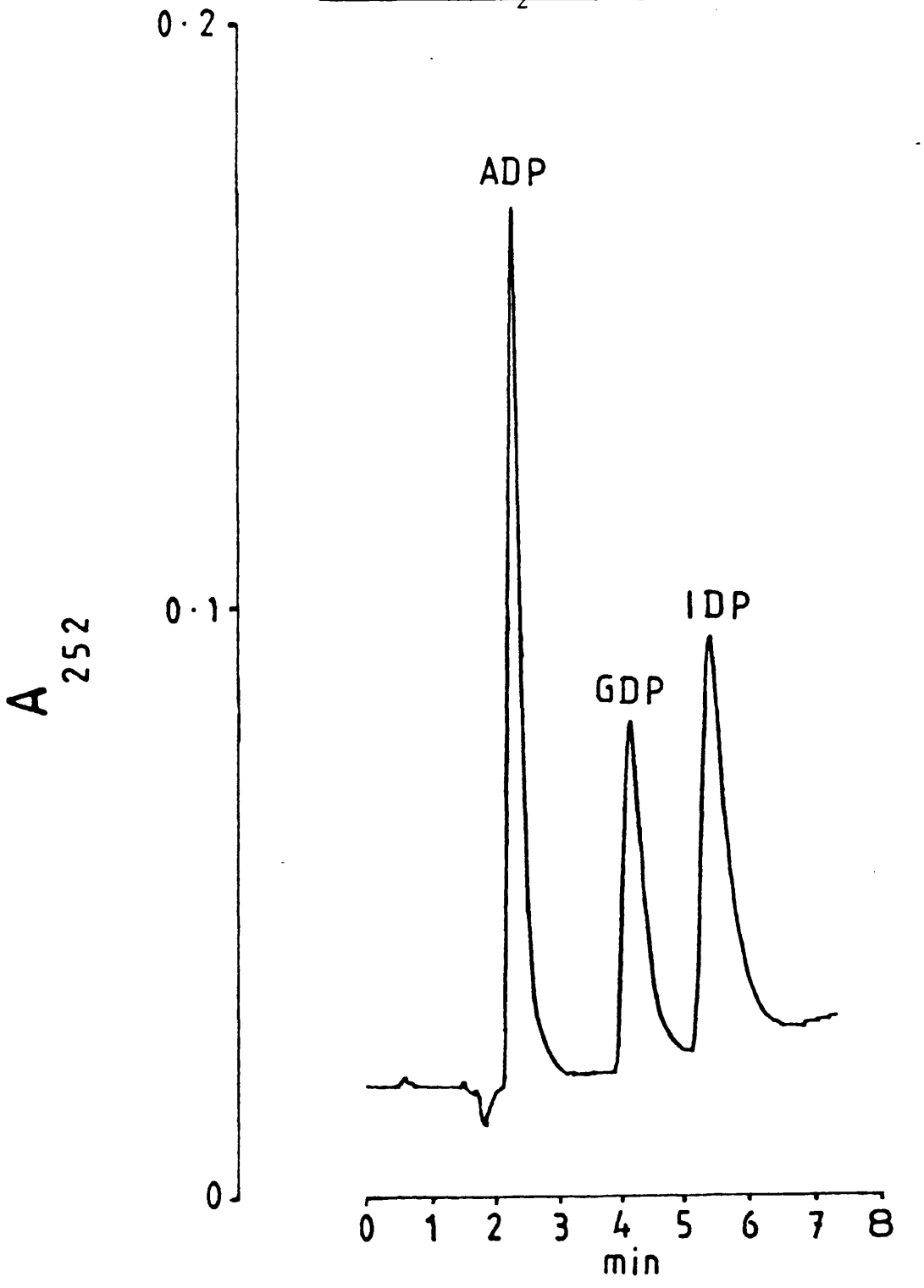
sample = 20 µl (10 mM) IDP

flow rate = 2 ml/min

scale = 0-2·0

chart recorder = 10 mm/min

Figure 15 HPLC analysis of a mixture of nucleotides on Zorbex-NH_2 column



sample = 20 µl [(3·3 mM) ADP, GDP & IDP]

flow rate = 2 ml/min

scale = 0-2·0

chart recorder = 10 mm/min

activity due to cross-contamination.

2·8 Bacterial & yeast cultures

2·8·1 Maintenance & growth of organisms

Bacterial strains and *S. cerevisiae* were maintained on nutrient-agar plates at 4°C and sub-cultured every two weeks. The culture of organisms occurred in nutrient broth media at 30°C or 37°C with shaking. Growth curves were followed by estimation of cell density by measurement of absorbance at 680 nm.

2·8·2 Culture media

Nutrient broth was used for the routine growth of organisms. When a specific carbon source was required, growth was carried out in a medium of basal salts : 50 mM NH_4Cl, 0·18 mM $CaCl_2$, 0·33 mM $MgSO_4$, 0·018 mM $MnSO_4$, 0·014 mM $FeSO_4$ in 50 mM sodium/potassium phosphate buffer, pH 7·2, with addition of the carbon source.

2·8·3 Gram stain

The Gram stain was performed by the method of Gillies & Dodds (1973). A small loop of bacteria to be examined was added to a drop of sterile saline on a microscope slide. After allowing the mixture to dry, the material was fixed by passing the slide three times through a bunsen burner flame. The slide was flooded with methyl violet (0·5% (w/v) in H_2O), left for 5 min, and then rinsed with Gram's iodine (0·3% iodine/0·6%

potassium iodide (w/v in H_2O). After a further 2 min the slide was drained and the cells decolourized by exposure to acetone for 5 s, before washing in H_2O. The counter-stain Neutral Red (1% Neutral Red + 1% acetic acid in H_2O) was then applied and after 30 s the slide was again rinsed with H_2O and gently blotted dry.

2·9 Isolation & culture of *Trypanosoma brucei*

2·9·1 Bloodstream cell form (strain MITat 1·1)

These were isolated from the blood of 250 g Wistar rats, 71 h after intraperitoneal injection with a dosage of 10^7 viable trypanosomes. The cells were separated from blood components by centrifugation (600 x g) for 10 min at 4°C and further purified on a short DEAE-Sephacel column (5 ml bed volume) in isotonic P.B.S., pH 8·0, containing 10 mM glucose as described by Lanham & Godfrey (1970). At pH 8·0 blood cells absorb to the column, whereas the less negatively charged *T. brucei* cells are eluted. Cell number and viability were monitored by phase contrast microscopy. Active trypanosomes were counted using a haemocytometer and x40 objective of a Zeiss microscope.

2·9·2 Procyclic cell form (strain EATRO-427)

These were cultured at 26°C on the very complex SDM-79 medium (Brun & Schonenberger, 1979) supplemented with 10% (v/v) fetal calf serum. Cultures were initiated by adding a volume of an established procyclic culture to

5 ml of complete SDM-78 medium, so that the final cell density was 10^6 cells/ml. Cultures may also be initiated using stabilates (procyclic cells in 20% glycerol stored in liquid nitrogen). Within 4 days the cultured cells reached a density of 1-5 x 10^7 cells/ml. At this point, to prevent exhaustion of available nutrients, cells may be diluted into fresh medium or harvested. Cells were harvested by centrifugation (600 x g) for 5 min at room temperature and then washed in 100 mM HEPES buffer, pH 7·5, containing 25 mM $NaHCO_3$, 50 mM NaCl, 5 mM KH_2PO_4, 5 mM L-proline, 5 mM KCl and 5 mM glucose; the cells were then stored on ice.

2·10 Induction of physiological dysfunction and metabolite measurements

2·10·1 Induction of diabetic ketoacidosis

Female Wistar rats (150-160 g), following 24 h starvation, were used. Diabetes was induced by intravenous administration (tail vein), under ether anaesthesia, of streptozotocin (150 mg / Kg body weight) dissolved in 50 mM sodium citrate buffer, pH 4·2, at 60 mg/ml. Control and treated animals were fed (C.R.M-labsure) *ad libitum* and given 5% (w/v) glucose in the drinking water for the initial 12 h.

2·10·2 Estimation of diabetic condition

24 and 48 h after treatment, animals were assessed as to the severity of diabetes by estimation of

urinary glucose and acetoacetate with Clinistix and Ketostix strips, respectively. Treated animals that failed to give a positive glucose test at 24 h and a positive acetoacetate test at 48 h, were removed from the experiment.

2·10·3 Insulin controlled diabetes

Commencing 24 h after treatment, diabetic animals received subcutaneous administrations of insulin (5 units /animal/day) until body weights approached control values. Individual animals that failed to increase in body weight in a given 24 h period, were given an additional 2 units of insulin for that day.

2·10·4 Determination of plasma glucose

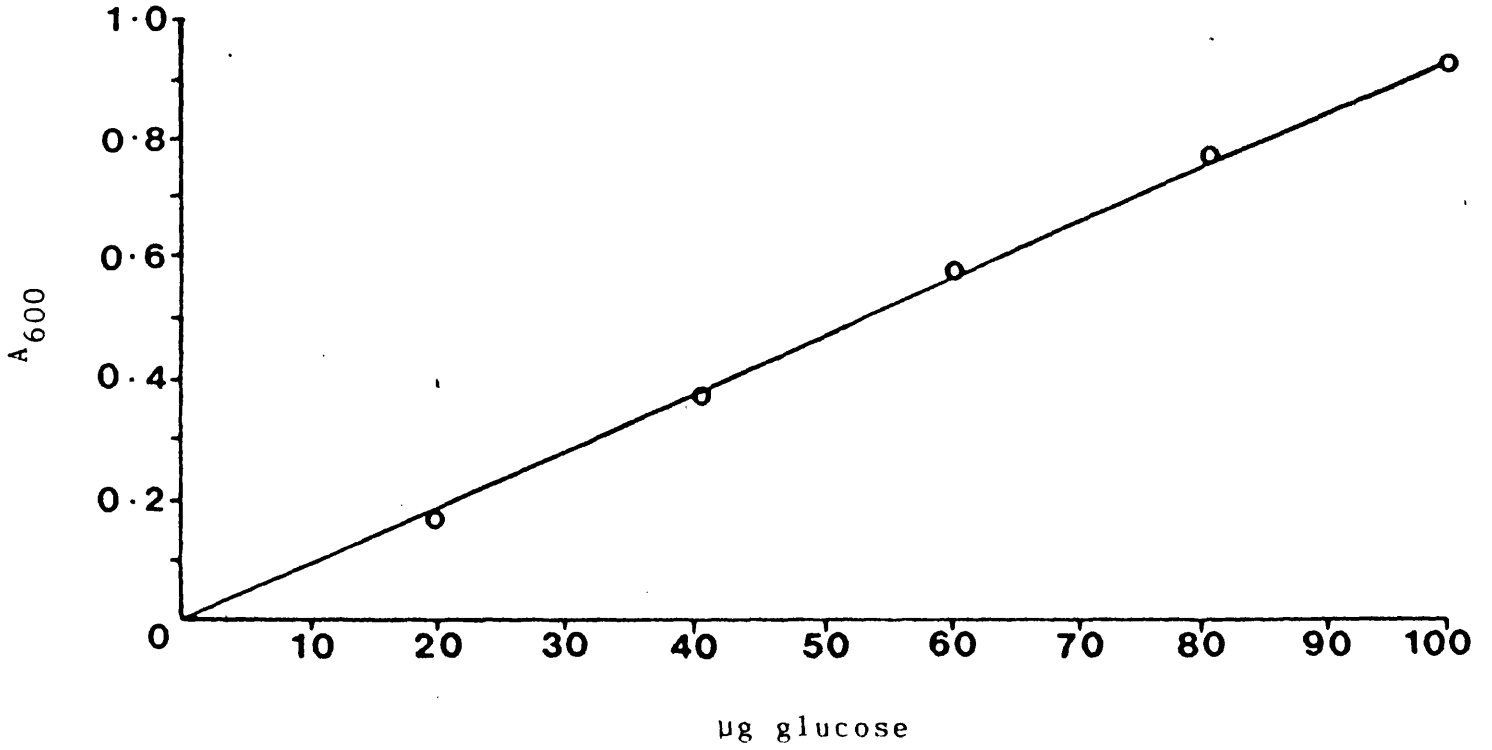
Immediately after decapitation, 0·1 ml of whole blood was added to 1·5 ml of H_2O and deproteinated by the addition of 0·2 ml of 0·3 M $Ba(OH)_3$ and 0·2 ml of 5% (w/v) $ZnSO_4$. Following centrifugation (3000 x g) for 15 min at 4°C the glucose concentration of the supernatant was determined by the method of Asatoor & King (1956). To 1 ml of the deproteinated sample was added 1 ml of copper reagent (0·6% (w/v) $CuSO_4.5H_2O$) and 1 ml of Harding's reagent (see Appendix I). The samples were thoroughly mixed and boiled for 20 min. After cooling, 1 ml of Nelson's reagent (see Appendix II) was added followed by 6 ml H_2O. After mixing, absorbances were recorded at 600 nm. A standard glucose curve (0-100 µg)

was prepared for each determination (see Figure 16). For diabetic animals an initial x 20 dilution was required.

2·10·5 Determination of plasma ketone bodies

Immediately after decapitation, 0·5 ml of whole blood was deproteinated by addition of 0·5 ml ice-cold 10% (w/v) perchloric acid. After thorough mixing and centrifugation (3000 x g) for 15 min at 4°C, the supernatant was collected and its volume recorded. Neutralization was quickly achieved by addition of 5 µl B.D.H.-universal indicator and slow addition of 4 M KOH until the indicator became green/blue (pH7-8). After 30 min on ice, samples were centrifuged (3000 x g) for 10 min at 4°C. Supernatant volumes were again recorded before enzymic determination of acetoacetate and β-hydroxybutyrate by the methods of Williamson & Mellanby (1974). The acetoacetate assay mixture contained: 0·2 mM NADH, 50 mU/ml β-HBDH and 300 µl test sample in 50 mM potassium phosphate buffer, pH 6·8, in a total volume of 1 ml. The decrease in absorbance at 340 nm was recorded after 10 and 20 min. The β-hydroxybutyrate assay mixture contained: 0·4 mM NAD^+, 50 mU/ml β-HBDH and 300 µl test sample in Tris-HCl/ hydrazine buffer, pH 8·5 (see AppendixIII). The increase in absorbance at 340 nm was recorded at 40 and 60 min. (ε_{340} = 6·22 x 10^3 $l.mol^{-1}cm^{-1}$).

Figure 16 Glucose standard curve



2·10·6 Induction of hepatic porphyria

Hepatic porphyria was induced by the drug 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC). Female mice (strain C.F.L.P.), each about 30 g and individually housed, were given drinking water *ad libitum* and starved for 24 h. Each mouse of the treatment group then received 5 g of lab chow (C.R.M.-Labsure) containing 10 mg DDC, the control group each received only 5 g of lab chow. Mice were sacrificed 24 h later and the livers were immediately removed. Aliquots (0·5 g) of control and treated livers were retained for total liver porphyrin determination, while the remainder of the livers were pooled into control and treated groups for the preparation of mitochondria.

2·10·7 Estimation of total liver porphyrins

Total liver porphyrins were estimated by the method of Abbritti & De Matteis (1972). The 0·5 g aliquots were homogenized with an Ultra-Turrax tissue homogenizer in 5 ml of H_2O. 0·5 ml of the homogenate was then added to 4·5 ml H_2O, followed by 0·5 ml of 10·2 M perchloric acid and 5 ml of absolute ethanol. After mixing and storage on ice, in the dark, for 15 min the mixture was centrifuged (3000 x g) for 15 min at 4°C. The supernatant was then allowed to warm up to room temperature, in the dark. The porphyrin content was determined fluorimetrically (excitation λ = 400 nm, fluorescence λ = 550-650 nm) in an Amico-Bowman

Spectrophotofluorimeter, using an internal mesoporphyrin standard. Results are expressed in nmol/g wet liver.

2·11 Preparation of mitochrondria

All procedures were carried out at 4°C, the sucrose /Tris extraction buffer contained: 0·25 M sucrose + 1 mM EDTA + 0·2 mM EGTA + 0·1% BSA (fatty acid free) in 50 mM Tris-HCl buffer, pH 7·5. Tissue : buffer ratio was 1 : 4.

2·11·1 Bovine heart mitochondria

Mitochondria were prepared by the method of Azzone *et al.* (1979), with slight modifications. Fresh heart tissue was diced into cubes and homogenized (1 g tissue : 2 ml extraction buffer) in a Waring blender, 10 s at low power, with ice-cold sucrose/Tris extraction buffer. 1 M Tris was used to maintain pH 7·5. A second homogenization, 15 s at medium power, was followed by centrifugation (1,600 x g) for 15 min. The supernatant was filtered through double-cheesecloth and centrifuged (20,000 x g) for 30 min at 4°C. The mitochondrial pellet was resuspended in sucrose/Tris buffer containing no BSA and centrifuged (20,000 x g) for 30 min. The resultant pellet was stored on ice.

2·11·2 Mouse liver or rat brain mitochondria

Fresh tissue was washed and diced into ice-cold sucrose/Tris extraction buffer. This was then gently hand-homogenized using a Potter/Dounce homogenizer with a

teflon plunger (gap = 0·31 mm) for 5 complete strokes. The homogenate was then centrifuged (1600 x g) for 10 min. After filtration through double-cheesecloth, the supernatant was centrifuged (30,000 x g) for 30 min. The mitochondrial pellet was then gently resuspended in sucrose/Tris buffer, containing no BSA, centrifuged (30,000 x g) for 30 min and stored on ice.

2·11·3 Locust flight muscle mitochondria

150 adult locusts were dissected and their flight muscles placed in 20 ml of sucrose/Tris extraction buffer. This was then hand-homogenized using a Potter-Dounce homogenizer with a glass plunger (gap = 0·25 mm) for 10 complete strokes. The homogenate was centrifuged (1000 x g) for 10 min and then passed through a fine nylon filter to remove fat granules, before centrifugation (30,000 x g) for 30 min. The small 'halo' of membrane material was removed from the main pellet, which was gently suspended in sucrose/Tris buffer, containing no BSA and centrifuged (30,000 x g) 30 min and stored on ice.

2·11·4 Zea Maize mitochondria

Etiolated and non-etiolated plant tissue was cut into 2 cm sections and suspended in Mannitol extraction buffer containing 0·3 M mannitol, 1 mM EDTA, 0·1% (w/v) BSA (fatty acid free) in 100 mM MOPS buffer, pH 7·5, and homogenized with an Ultra-Turrax homogenizer for 3 s on ice. The supernatant was squeezed through 4-layers of

double-cheesecloth and then centrifuged (1600 x g) for 15 min. The supernatant was centrifuged (30,000 x g) for 30 min. The resultant mitochondrial pellet was gently resuspended in Mannitol/MOPS extraction buffer containing no BSA, centrifuged (30,000 x g) for 30 min and stored on ice.

2·11·5 Preparation and isolation of intact mitochondria using Percoll(bovine heart)

Percoll was made isotonic by diluting with 2·5 M sucrose (9 : 1). Discontinuous gradients were composed of 13·5, 28 and 55% (15 ml each) Percoll, carefully prepared in thin-walled 50 ml polyallomer (Sorvall) centrifugation tubes. The gradients were stored at 4°C until required. Crude mitochondrial preparations (20 mg protein/centrifugation tube) were gently layered onto the gradients, which were then centrifuged (12,000 x g) for 1 h at 4°C. Intact mitochondria were found to band at the 28/55% interface. Fractionation was achieved using an MSE gradient fractionator. After establishing an air-tight seal, a hollow needle was inserted through the bottom of the tube and by means of a finely controlled air valve slowly fractionated the gradient. Mitochondrial integrity was assessed by the latency of citrate synthase actively with and without 0·05% Triton X-100 in the assay mixture.

2·12 Methods of cellular & mitochondrial disruption

2·12·1 French press

Yeast cells at 1 g/ml of 100 mM Tris-HCl + 1 mM EDTA buffer, pH 7·5 were disrupted by passage twice through a pre-cooled French press cell at a pressure of 62 MPa (9000 lb/in^2)

2·12·2 Ultrasonication

Ultrasonication of bacteria or mitochondria was achieved using a MSE 100 W sonicator, with a 5 mm probe, operated at 40 W for 6 x 15 s with cooling.

2·12·3 Tissue homogenization

Tissue homogenization was achieved using an Ultra-Turrax tissue homogenizer (5 x 15 s bursts interspersed with cooling). Ossified tissues, such as bone marrow, were homogenized using a Sorvall Omni-mixer (4 x 15 s bursts interspersed with cooling, at full power).

2·12·4 Osmotic lysis

Pelleted mitochondria were gently resuspended in ice-cold 20 mM Tris-HCl buffer, pH 7·5, containing 20% (v/v) glycerol and incubated for 30 min at 4°C.

2·12·5 Triton X-100

Pelleted mitochondria were resuspended in 20 mM Tris-HCl buffer, pH 7·5, containing 0·05% (v/v) Triton X-100 and incubated for 10 min at 4°C.

2·12·6 Toluenization

Yeast cells were made permeable to low molecular weight substances by the method of Weitzman (1973). Cells were washed with 100 mM Tris-HCl buffer, pH 8·0, resuspended at 0·1 g cell/ml buffer and prewarmed to 37°C. 50 µl of toluene/ethanol mixture (1 : 4 (v/v)) was added per ml of extract which was then shaken vigorously for 5 min at 37°C. After cooling on ice, the cells were centrifuged (600 x g) for 2 min at 4°C and resuspended in fresh 100 ml Tris-HCl buffer, pH 8·0. This washing procedure, to remove toluene, was repeated 6 times. Finally the cells were resuspended at 0·1 g cell/ml buffer and stored on ice.

2·13 Centrifugation

Differential centrifugations were performed in a Sorvall RC 5B super-speed centrifuge (Du Pont Instruments), Beckman L5-50B Ultracentrifuge and Eppendorf microfuge. Maximum relative centrifugal force (g max) was calculated from r.p.m. values as follows:

$$RCF = 1{\cdot}12 \times 10^{-5} \cdot R \cdot N^2$$

where R = radius (cm) from the centre of the rotor shaft to the tip of the centrifuge tube

N = r.p.m.

2·14 Polyacrylamide gel electrophoresis (PAGE)

2·14·1 Non-denaturing PAGE

Non-denaturing gels (7% (w/v) acrylamide) were prepared according to the method of Davis (1964). The resolving gel buffer was 0·37 M Tris-HCl, pH 8·9, the stacking gel (3% acrylamide); the buffer was 0·12 mM Tris-HCl + 0·19 M glycine, pH 8·0. Ammonium persulphate was used as the initiator of the acrylamide/bis-acrylamide polymerization, with TEMED as the catalyst. Depending upon the staining to be employed, 2-20 µg protein samples were applied to each gel in 50 µl of reservoir buffer containing 10% (w/v) sucrose + 0·001% (w/v) bromophenol blue. The samples were run at 0·5 mA/gel until the dye bands had traversed the stacking gel; the current was then increased to 2 mA/gel for the remainder of the run.

2·14·2 SDS - PAGE

SDS-gels (5% (w/v) acrylamide) used for estimation of sub-unit molecular weight, were prepared according to the method of Weber & Osborn (1969). The reservoir and gel buffer were 0·1 M sodium phosphate buffer, pH 6·7, + 0·1% (w/v) SDS. Polymerization was achieved using ammonium persulphate and TEMED. 2-20 µg protein samples were dissolved in 40 µl of 0·1 M sodium phosphate buffer, pH 6·7, 0·1% (w/v) SDS, 10% (v/v) glycerol, 0·2 M 2-mercaptoethanol and 0·001% (w/v) bromophenol blue, boiled for 2 min and then loaded.

Samples were run into the gel at 2 mA/gel and then at 8 mA/gel for the remainder of the run.

2·14·3 Staining of polyacrylamide gels

Samples containing more than 5 µg protein were stained with Coomassie Brilliant Blue (CBB). Gels were fixed overnight in 50% (v/v) methanol + 10% (v/v) acetic acid, followed by staining for 45 min at 37°C in 0·1% (w/v) CBB + 50% (v/v) methanol + 10% (v/v) acetic acid. Gels were then destained in 5% (v/v) methanol + 7·5% (v/v) acetic acid at 37°C.

Samples containing 1-5 µg protein were stained using the silver staining method of Morrisey (1981), all procedures being carried out at 37°C. Gels were incubated for successive 30 min incubations in 50% (v/v) methanol + 10% (v/v) acetic acid, then 5% (v/v) methanol + 7% (v/v) acetic acid and finally 10% (v/v) glutaraldehyde. The gels were then washed overnight in distilled H_2O (500 ml/gel). The following day the gels were incubated for 30 min in 5 µg/ml DTT. This was then removed (no rinsing) and 0·1% (w/v) $AgNo_3$ added. After 30 min the gels were rapidly rinsed with distilled H_2O and covered with 15 ml of developer (50 µl of 37% (v/v) formaldehyde in 100 ml of 3% Na_2CO_3). Staining was terminated by adding 0·75 ml of 2·3 M citric acid to each gel. The gels were washed (x 5) with distilled H_2O and soaked in 0·3% Na_2CO_3 for 10 min. Finally the gels were washed in distilled water and stored in the dark.

2·15 Gel chromatography

2·15·1 Preparation and use of Gel filtration Matrices

A column (2·5 x 35 cm) of Sephacryl S-200 (supplied preswollen) was used to determine the approximate size of STKs (ie large or small type) from animal and bacterial extracts. The column was equilibrated in 0·1 M sodium/potassium phosphate, pH 8·0, at 4°C (with the inclusion of 20% (v/v) glycerol for the bacterial extracts). 50 µl of LDH (0·25 mg) was added as a marker to the test sample, to distinguish between large and small STKs. Before application, the sample was made 10% (w/v) with respect to sucrose, to increase the density. 2-ml fractions were then collected.

Sephadex G-10 (swollen for 3 h at room temperature) was used for desalting samples and separation of succinate from succinyl-CoA.

Prepacked P-10 columns (Sephadex G-25) were also used to desalt samples up to 2 ml in volume. Sepharose 4B, as used in affinity and dye chromatography, was supplied preswollen.

2·15·2 Hydroxylapatite Columns

Hydroxylapatite (Bio-Rad HTP) was resuspended (1 : 6 v/v) in 10 mM sodium/potassium phosphate buffer, pH 7·5. The matrix was repeatedly resuspended and fined. Columns were poured and allowed to settle under gravity. The loading capacity was 5 mg protein/ml bed volume, with a maximum flow rate of 12 ml/h. Elution was achieved by

a linear phosphate gradient. The void volume was calculated to be ~ 70% of the bed volume.

2·15·3 Affinity chromatography

2·15·3·1 CNBr activation of Sepharose 4B

This was achieved by the method of Marsh *et al.* (1974). To a slurry of Sepharose 4B (4 ml of packed gel + 4 ml H_2O) was added 8 ml of 2 M Na_2CO_3 and the mixture slowly stirred. The stirring rate was increased and 1 ml CNBr/acetonitrile solution (0·8 g CNBr/ml) was added dropwise. After 2 min vigorous stirring the slurry was washed on a coarse sintered-glass filter with 50 ml of 0·1 M Na_2CO_3 pH 9·5, followed by 50 ml of H_2O.

2·15·3·2 Preparation of a dial-2',3'-nucleoside diphosphate affinity column

This was achieved by the method of Ball & Nishimura (1980). 16 ml of packed CNBr-Sepharose 4B were washed with 1 l of 1 mM HCl. A spacer arm (C8) was then covalently attached by stirring the gel very gently with 17·5 ml of 0·1 M Na_2CO_3, pH 9·5, containing 1·57 g of adipic acid overnight at 4°C. The following day the nucleoside diphosphate (ADP, GDP or IDP) was peroxidated by adding 2·5 ml of NDP (10 mg/ml) to 2·5 ml of sodium meta periodate (4·3 mg/ml) and stirring for 1 h in the dark at 4°C. The adipic acid-linked Sepharose 4B was washed with 1 l of water and 1 l of 0·2 M NaCl and then stored in 10 ml of 0·1 M sodium acetate, pH 5·0, at 4°C.

5 ml of the NDP/periodate mixture were then added to the gel, followed by 25 ml of 0·1 M sodium acetate, pH 5·0, and stirred gently for 3 h at 4°C. 75 ml of 2 M NaCl were then added to the mixture which, after a further 30 min, was washed with 1 l of water, and stored at 4°C. For best results the gel was used within 2 days of synthesis.

2·15·4 Preparation of triazine dye columns

6 ml columns were prepared from 15 supplied triazine dyes:

1. Red MX-5O	2. Red HE-3B	3. Ramazol Red
4. Red R3BN	5. Scarlet MXG	6. Brown H2G
7. Brown MX-5BR	8. Blue MX-3G	9. Blue HE-RD
10. Blue PGR	11. Turquoise H-A	12. Turquoise H-7G
13. Green HE4BD	14. Yellow MX G8	15. Orange MX 2R.

All coupling procedures were carried out at room temperature. To 6 ml aliquot of Sepharose 4B (thick slurry) were added 17·5 ml of water followed by 60 mg of dye dissolved in 5 ml of water. The mixtures were stirred for 10 min before the addition of 2·5 ml of 20% (w/v) NaCl, and then stirred for 30 min. 0·31 ml of 5 M NaOH was then added and the mixtures stirred gently for 3-4 days. The gels were then washed, to remove unbound dye, with successive 50 ml-washes of water, 1 M NaCl and 6 M urea. The gels were then stored at 4°C in 20 mM Tris-HCl + 2 mM $MgCl_2$ buffer, pH 7·2 containing 0·01% sodium azide until required.

2·15·5 Fast Protein Liquid Chromatography (FPLC)

The Pharmacia FPLC system was used. The system comprised a GP-250 gradient programmer, two P-500 pumps, gradient mixer, V-7 valve, single path UV-1 monitor and control unit, Frac-100 fraction collector and chart recorder. Samples (up to 10 ml) were loaded through a superloop onto a Mono Q (anion exchanger) column which had previously been equilibrated with 10 column volumes of 20 mM Tris-HCl + 2 mM $MgCl_2$ buffer, pH 7·5. The column was eluted with a KCl gradient and protein was monitored at 280 nm with a UV source connected to a microprocessor-controlled fraction collector. Absorbance sensitivities of 0·2-2·0 were used. Figure 17 shows the FPLC gradient programme used in the purification of pigeon A-STK. The FPLC system was also used with a Superose-12 (gel filtration) column for estimation of Mr. The column was equilibrated with 0·1 M Tris-HCl buffer, pH 8·0, containing 20% (v/v) glycerol and was run at 0·3 ml/min.

2·16 Ultrafiltration

The concentration of column fractions was achieved by using an Amicon Ultrafiltration cell (50 ml) (Amicon Corporation, U.S.A.) operated at 64 psi with oxygen-free nitrogen. The procedure was carried out at 4°C with a P10 membrane (mwt cutoff 10,000).

Figure 17 FPLC Programme method

Pharmacia Fine Chemicals

Laboratory record

Sample: Pigeon breast A-STK (after dial-ADP-column)

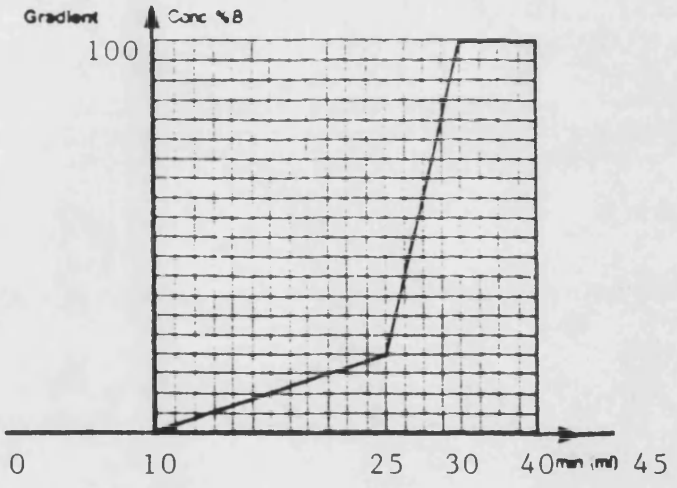
Gel	Column size	Temp
Mono Q	bed size 5 x 50 mm	25 °C

Eluent: 20 mM Tris-HCl + 2 mM $MgCl_2$ + 1 M KCl pH 7·5

Flow	Pressure	
1 ml/min	1·5	psi bar MPa

Sample size	
mg/ml	2 ml µl (ml)

Detector	Sensitivity	Chart speed
U.V.	0-0·5	1 cm/min (ml)



Method

Time (vol)	Instruction	Value	Note
0	conc % B	0	
0	ml/min	1	
0	cm/ml	1	
0	Port.Set	6·1	6·1 starts the fraction collector
10	conc % B	0	
25	conc % B	20	
30	conc % B	100	
40	conc % B	100	
40	conc % B	0	
45	conc % B	0	
45	Port.Set	6·0	6·0 stops the fraction collector

CHAPTER 3

EUKARYOTIC SUCCINATE THIOKINASES

CHAPTER 3

EUKARYOTIC SUCCINATE THIOKINASES

3·1 STK activities from mammalian & non-mammalian sources

The eukaryotic STK enzyme that has received most attention is the pig heart enzyme. Originally, when the succinate thiokinase reaction was first proposed and identified in pig heart extracts (Kaufman, 1951), the enzyme was considered to be linked to adenine nucleotides. However, it was later reported (Sanadi *et al.*, 1956) that after removal of nucleoside diphosphokinase activity, pig heart STK showed strict specificity for guanine and inosine nucleotides (G-STK). These, and similar findings of other guanine-linked animal STKs, led to the view that all animal STKs were guanine nucleotide-specific enzymes (Bridger, 1974), a situation generally presented in most biochemistry textbooks. Some years ago, however, an adenine nucleotide-linked enzyme (A-STK) was reported to occur in blowfly flight muscle (Hansford, 1973) and, more recently, A-STK activities have been found in a wide range of animals (McClellan & Ottaway, 1980), though not in mammalian tissues. Interestingly, Steiner & Smith (1981) presented immunological evidence for the existence of an A-STK in rat brain mitochondria. Antibody raised to pig heart G-STK was shown to cross-react with a brain G-STK and what appeared to be a brain A-STK. However, no enzyme activities were demonstrated and the authors were unable

positively to identify the ATP-dependent phosphorylation they observed as due to autophosphorylation of an A-STK rather than an endogenous protein kinase.

Recently, work in Professor P.D.J. Weitzman's laboratory has shown that considerable variation occurs in the nucleotide specificity of a wide range of bacteria (Weitzman & Jaskowska-Hodges, 1982). Each organism appears to follow a taxonomic pattern in the nucleotide specificity of its STK. In the light of these results, and those previously mentioned, it seemed pertinent to re-examine carefully the nucleotide specificity of animal STKs (mammalian & non-mammalian) for the possible occurence of both G-STK and A-STK enzymes.

Rat tissues were homogenized with an Ultra-Turrax homogenizer as described. The resultant supernatants were used to assay for both guanine & adenine nucleotide-linked STK activities. The results in Table 2 indicated the existence of both G-STK and A-STK in rat tissues. Significantly, the ratio of G-STK to A-STK activities varied between tissues. A spectrum of ratios was found, G-STK being the predominant enzyme in kidney and liver, more nearly matching the A-STK in heart, and being exceeded by A-STK activity in brain and skeletal muscle. It is difficult to reconcile the approximate 10-fold change in the G-STK/A-STK ratio with the presence of a single STK enzyme of 'loose' specificity; one would have to postulate that the 'looseness' of specificity itself varied between tissues. A simpler explanation is that

Table 2 STK activities in extracts of various rat tissues

Tissue	G-STK activity ($nmol.min^{-1}.mg^{-1}$)	A-STK activity ($nmol.min^{-1}.mg^{-1}$)	Ratio G-STK/A-STK
kidney	6·9	2·0	3·5
liver	4·5	1·5	3·0
heart	3·6	2·5	1·4
brain	0·95	1·5	0·63
skeletal muscle	0·42	1·1	0·38

Tissues from 5 animals were pooled, homogenized (Ultra-Turrax) in 0·1 M sodium/potassium phosphate buffer, containing 1 mM EDTA, pH 8·0 and centrifuged (30,000 x g for 30 min at 4°C). Supernatants were then used without further treatment.

there are two, nucleotide-specific, STKs whose relative proportions differ between tissues. Interestingly, from Table 2, it is apparent that it is the G-STK which varies considerably in specific activity, whereas A-STK remains fairly constant throughout the tissues. McClellan & Ottaway (1980), who reported A-STK and G-STK in a number of non-mammalian animal tissues, also observed divergence in the G-STK/A-STK ratios and commented that this may be an indication of the presence of two enzymes, but no further experimental evidence was presented.

Subsequent studies on STK activities from other mammalian sources (Table 3) also indicated the presence of both G-STK and A-STK activities. It is conceivable that the apparent A-STK activity, in Tables 2 & 3, was due to a guanine nucleotide-specific STK coupled to the activity of nucleotide diphosphokinase (NDPK) and the presence of sufficient substrate (i.e. GTP). However, the apparent A-STK activity could only equal and never exceed the tissue's G-STK activity. Therefore, the fact that A-STK is the major activity in brain and skeletal muscle excludes the possibility that A-STK results from the action of NDPK.

G-STK and A-STK activities were also observed in other animal tissues (Table 4). Two mammalian cell lines contained both activities and in the case of the Baby hamster kidney cell line (BHK) the ratios of G-STK / A-STK was similar to that found in kidney tissue extracts. For

Table 3 STK activities from various mammalian sources

Source	Tissue	G-STK activity ($\mu mol.ml^{-1}.min^{-1}$)	A-STK activity ($\mu mol.ml^{-1}.min^{-1}$)	Ratio G-STK/A-STK
Bovine				
	heart	0·32	0·23	1·4
	bone marrow (sternum)	0·02	0·003	6·7
Porcine				
	brain	0·2	0·3	0·66
	heart	0·36	0·3	1·2
	liver	1·3	0·5	2·6
	kidney	0·66	0·2	3·3
Ovine				
	brain	0·05	0·1	0·5

Extracts were prepared as in Table 2, page 80.

Table 4 STK activities from various animal sources

Sample	G-STK activity ($nmol.min^{-1}.mg^{-1}$)	A-STK activity ($nmol.min^{-1}.mg^{-1}$)	Ratio G-STK/A-STK
Cell lines			
BHK (Baby hamster kidney)	2·0	0·8	2·5
L1210 (mouse lymphoma)	1·6	1·6	1·0
animal tissues			
pigeon breast muscle (40-60%$(NH_4)_2SO_4$	0·7	13·0	0·05
locust thorax	1·2	5·6	0·21

Cultured cells were disrupted by Ultrasonication and centrifuged (30,000 x g for 30 min at 4°C). Tissue extracts were prepared as in Table 2, page 80.

the first time G-STK activity was detected in pigeon breast muscle and locust thorax. Previously, both were thought to contain only A-STK activity (McClellan & Ottaway, 1980). The G-STK activity from pigeon breast muscle could not be detected in crude extracts and was only measurable in the 40-60% $(NH_4)_2SO_4$ cut.

3·2 Evidence for distinct G-STK & A-STK enzymes

3·2·1 Additivity of STK activities

Apart from observing a wide variation in the G-STK/A-STK ratio in tissues (Tables 2 & 3), another indication for the existence of two distinct enzymes, as opposed to a single STK with broad specificity, is provided by a comparison of the activities measured in the presence of ADP and GDP singly with that measured in their joint presence. A single enzyme saturated with the 'preferred' nucleotide would not show increased activity on addition of the second nucleotide. On the other hand, if two enzymes were present, saturation of one of them with its specific nucleotide should still permit the other enzyme to display activity on the addition of the other nucleotide; hence some additivity of activities should be observed. McClellan & Ottaway (1980) used additivity of the activities of STK with GTP and ATP, as a criterion for genuine utilization of both nucleotides (rather than an artefactual display of A-STK activity due to NDPK activity), though not for the existence of distinct STKs. Table 5 displays results that indicate additivity of A-STK and G-STK enzymes in extracts from porcine brain and bovine heart tissue, as well as mitochondrial extracts from locust flight muscle and bovine heart. The additivities observed were not strictly the sum of the two activities, but partial additivities (typically between 80-90%) were seen. A possible explanation for partial additivity is that, although each

Table 5 Additivity of STK activities for animal extracts

Sample	+ ADP	+ GDP	+(ADP + GDP)	% Additivity
	activity ($nmol.ml^{-1}.min^{-1}$)			
porcine brain				
(tissue extract)	98·2	49·1	120	81·5
locust flight muscle				
(mitochondrial extract)	553	166	617	85·8
bovine heart				
(tissue extract)	51	68·9	103	85·9
(mitochondrial extract)	330	736	920	86·3

Mitochondrial extracts were prepared by Ultrasonication and centrifugation (30,000 x g for 30 min at 4°C).
Tissue extracts were prepared as in Table 2 , page 80.

STK may be specific for one nucleotide as substrate, the other nucleotide may be able to act as a competitive inhibitor.

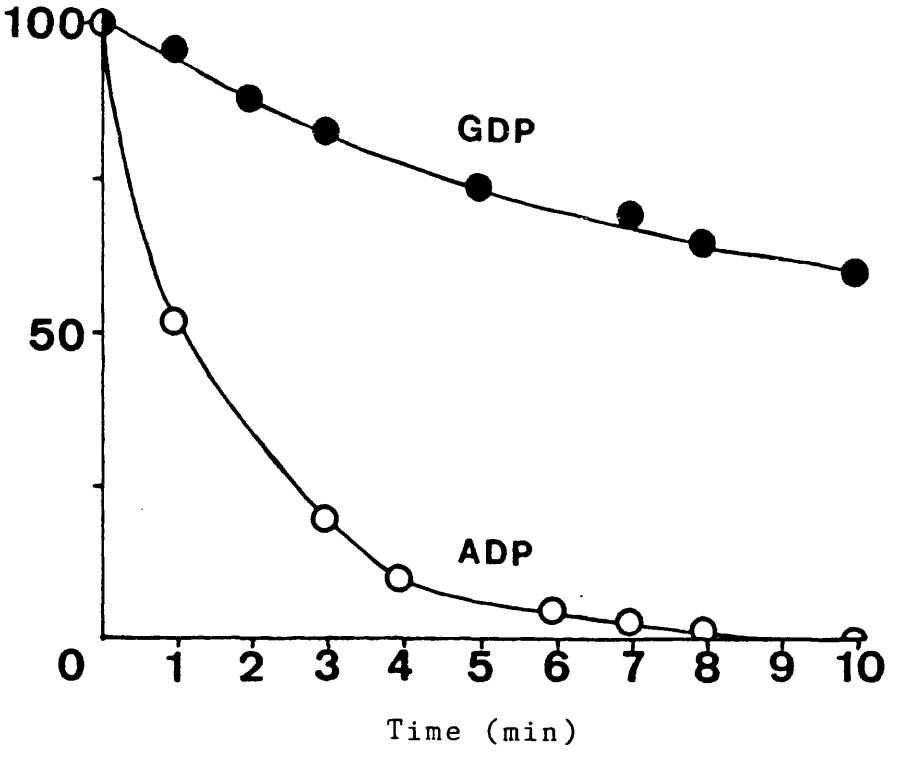
3·2·2 Thermal inactivation of STK activities

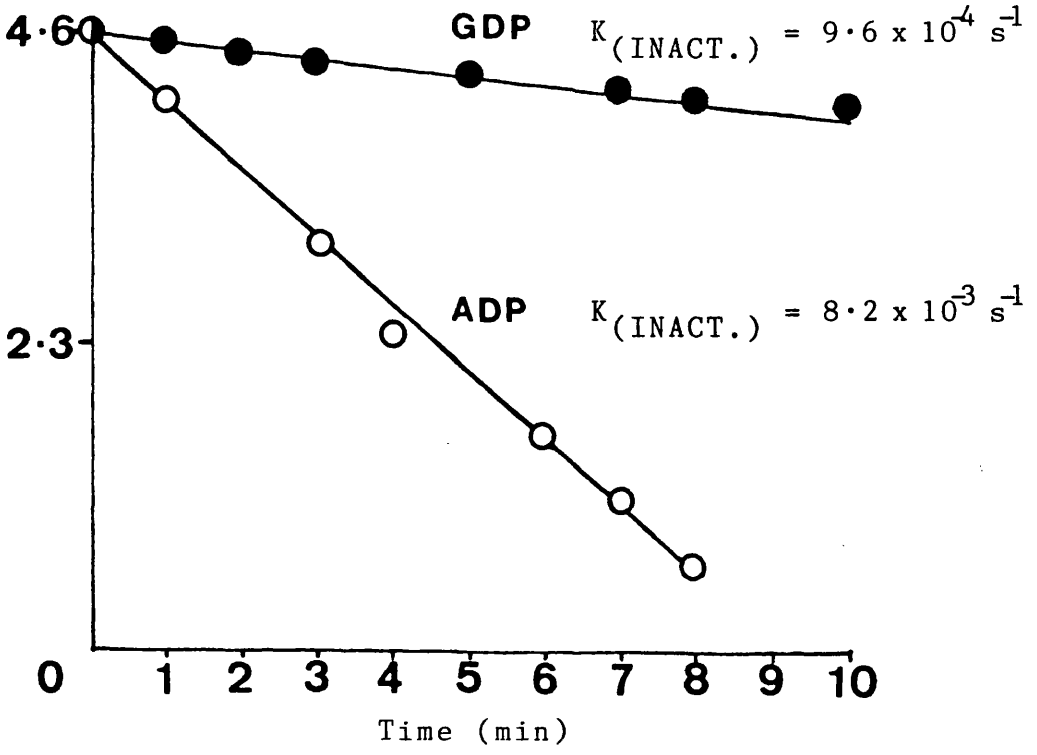
Additional evidence for two STKs was obtained from thermal inactivation studies. In rat heart extracts, and to a lesser extent in pig heart extracts, A-STK was shown to be more rapidly inactivated at 48°C, than G-STK (Figs. 18 & 19). Further stability studies were carried out on STK activities from bovine heart mitochondria. The mitochondria were resuspended in 20 mM Tris-HCl buffer, pH 7·5, containing 1 mM EDTA, sonicated and centrifuged (30,000 x g) for 30 min at 4°C. The supernatants were assayed for STK activities and then stored for 24 h at 4°C. G-STK only lost 4-5% over the 24 h period, whereas A-STK lost between 50-60% activity.

3·2·3 Separation of G-STK and A-STK enzymes

Conclusive evidence for distinct enzymes was obtained by separation of the two activities using affinity chromatography. This was carried out using derivatized GDP immobilized on Sepharose 4B, as described in Methods section 2·15·3·2. The separation of G-STK and A-STK activities was achieved with bovine heart mitochondrial extracts and locust flight muscle mitochondrial extracts (see Figs. 20 & 21). After loading with mitochondrial extract (0·5 ml) the column, equilibrated with 20 mM Tris-HCl buffer, pH 7·5, containing 2 mM $MgCl_2$, was washed with

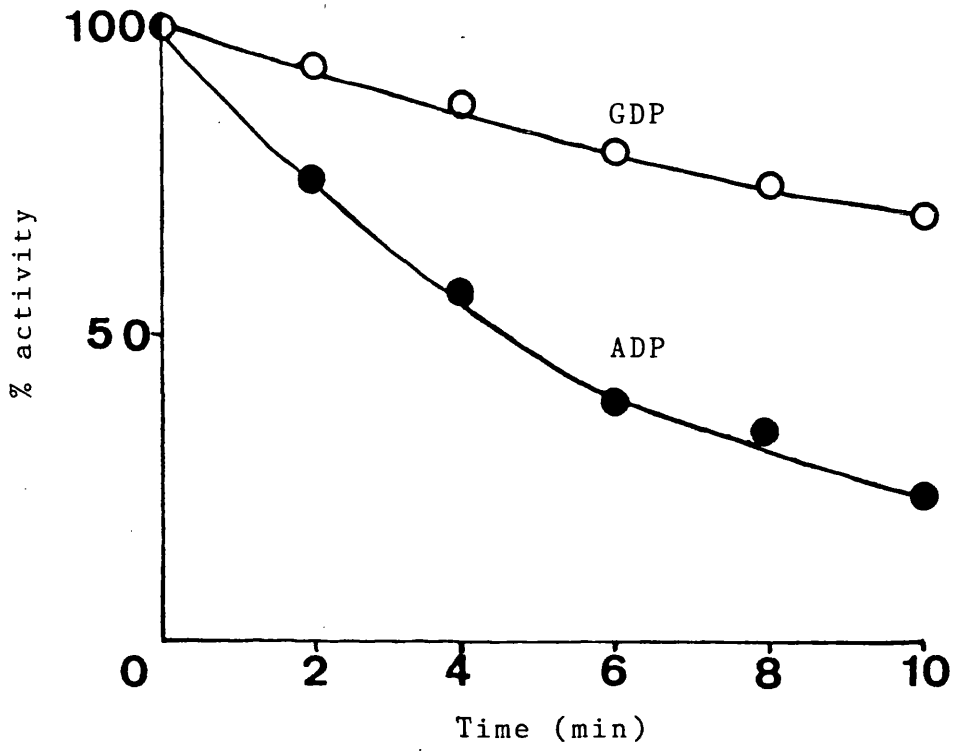
Figure 18 Thermal inactivation of rat heart STKs at 48°C

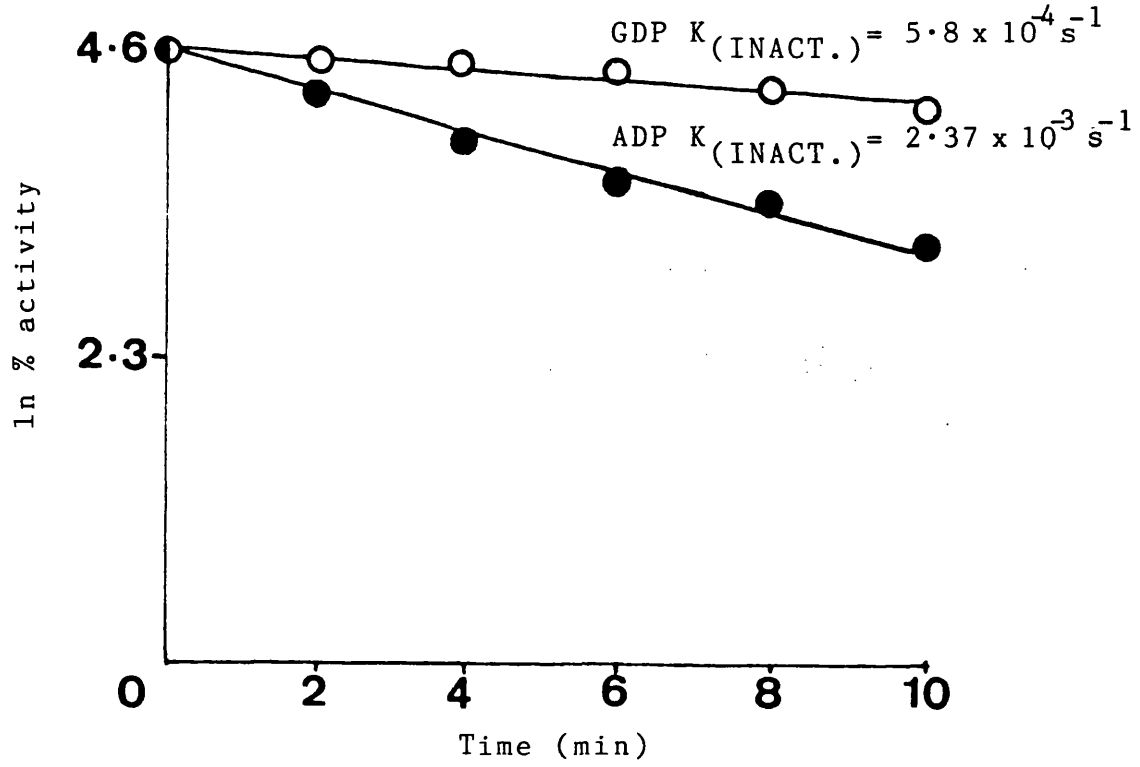




Extracts were prepared as in Table 2, page 80. 2·7 ml of 0·1 M sodium/potassium phosphate buffer, containing 1 mM EDTA, pH 8·0 was equilibrated at 48°C. At zero time, 0·3 ml of tissue extract was added and aliquotes (0·3 ml) were removed at various time intervals and assayed.

Figure 19 Thermal inactivation of pig heart STKs at 48°C





Experimental details were identical to Figure 18, page 88.

Figure 20 Separation of G-STK and A-STK activities from bovine heart mitochondria, on a 2',3'-dialdehyde-GDP affinity column, at 4°C

(15 x 150 mm)

Extracts were prepared as in Table 5, page 86.

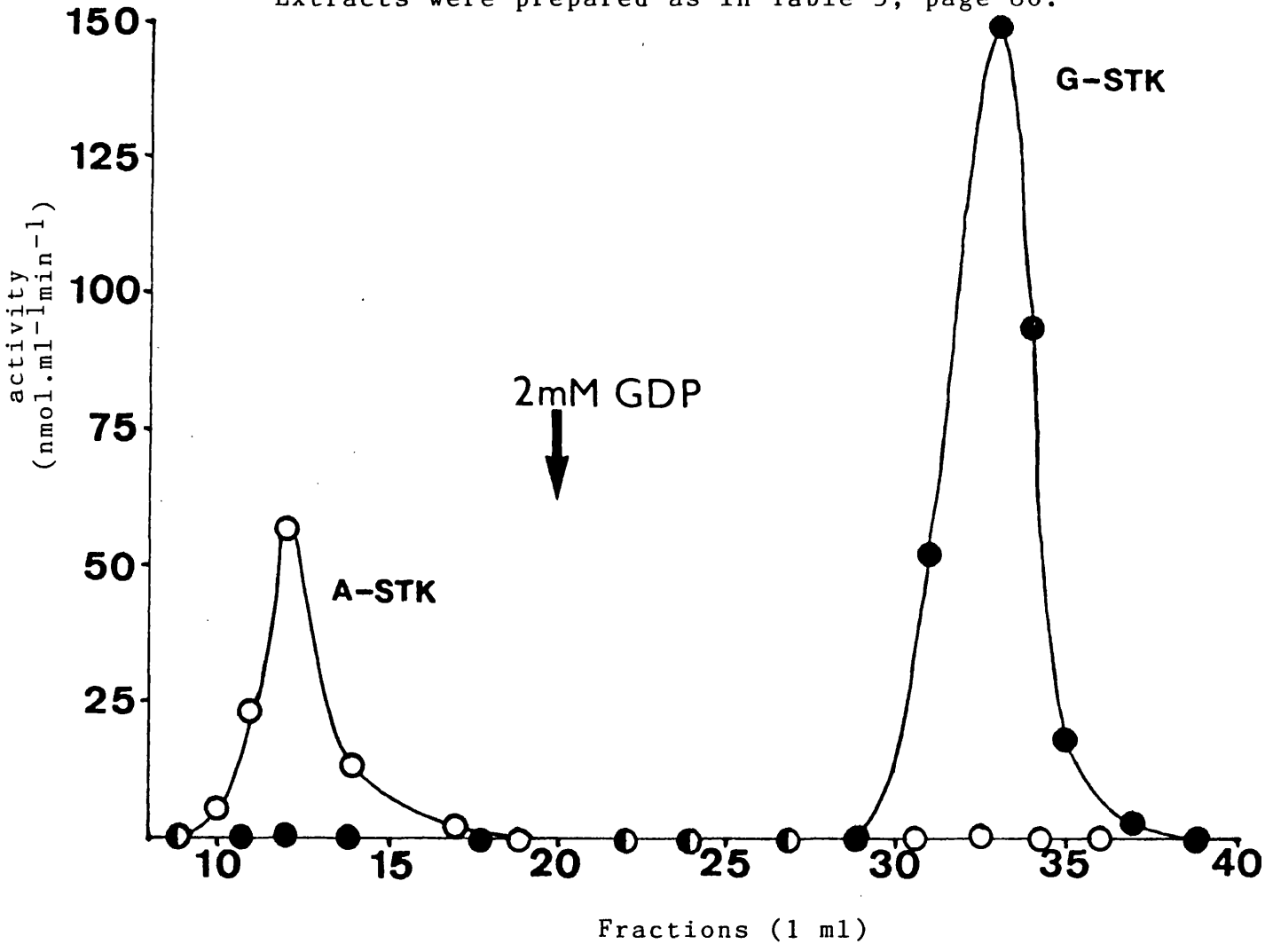
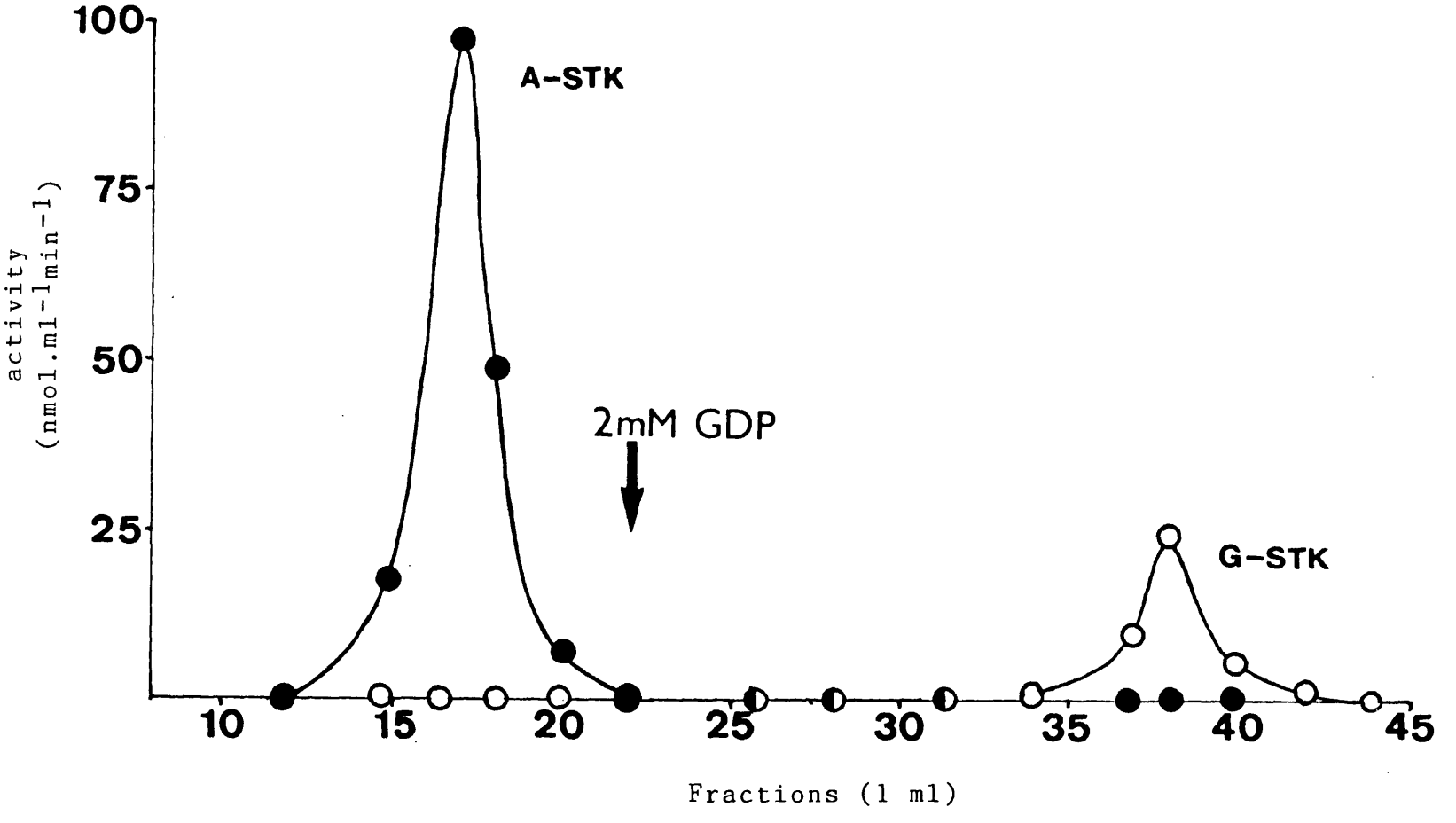


Figure 21 Separation of G-STK and A-STK activities from locust flight muscle mitochondria, on a 2',3'-dialdehyde GDP affinity column, at 4°C (15 x 160 mm)

Extracts were prepared as in Table 5, page 86.



equilibrating buffer and 1-ml fractions were collected. When the protein content of the eluted fractions had fallen to zero, the elution buffer was changed to 20 mM sodium/potassium phosphate buffer, pH 7·5, containing 2 mM GDP + 2 mM $MgCl_2$. Removal of GDP from the subsequent fractions, apparently containing STK activities, was achieved by gel filtration on a prepacked PD-10 column. Significantly, neither of the separated enzymes showed any activity whatsoever for the other nucleotide. Table 6 shows that, in the case of the separated STKs from bovine heart, the non-substrate nucleotide acts as an inhibitor of the 'other' activity; this inhibition therefore supports the explanation given above for partial additivity in Table 5, when activity measurements were made in the joint presence of both nucleotides.

The Ki of the bovine heart mitochondrial G-STK for ADP was determined from a secondary replot of the slopes of Lineweaver-Burk plots for various ADP concentrations. A Ki value of 233 µM ADP was obtained, this displayed a competitive type inhibition with respect to GDP (Fig. 22a & b).

Table 6 Specificity of separated bovine heart STKs, and inhibition by the non-substrate nucleotide

Enzyme	+ ADP	+ GDP	+(ADP + GDP)	Inhibition
	activity ($nmol.min^{-1}.ml^{-1}$)			(%)
A-STK	38·0	0	25·5	33
G-STK	0	99·5	65·5	34

Fig. 22a Lineweaver-Burk plot:

ADP inhibitor concentrations;

○- zero, ●- 100 µM,

□- 200 µM, ■- 300 µM.

Fig. 22b The intercept on the abscissa gives a Ki value of 233 µM ADP for the G-STK enzyme from bovine heart mitochondria

Figure 22a Linweaver-Burk plot: V^{-1} versus $[GDP]^{-1}$ for G-STK from bovine heart mitochondria with varying [ADP]

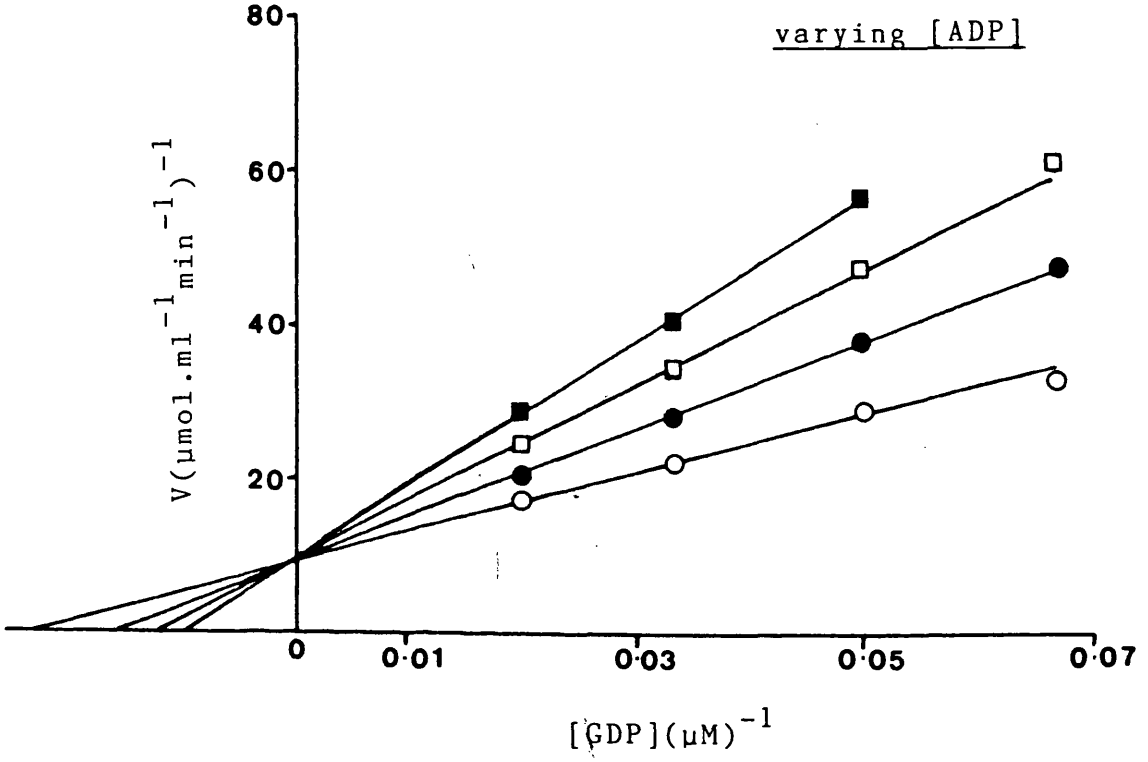
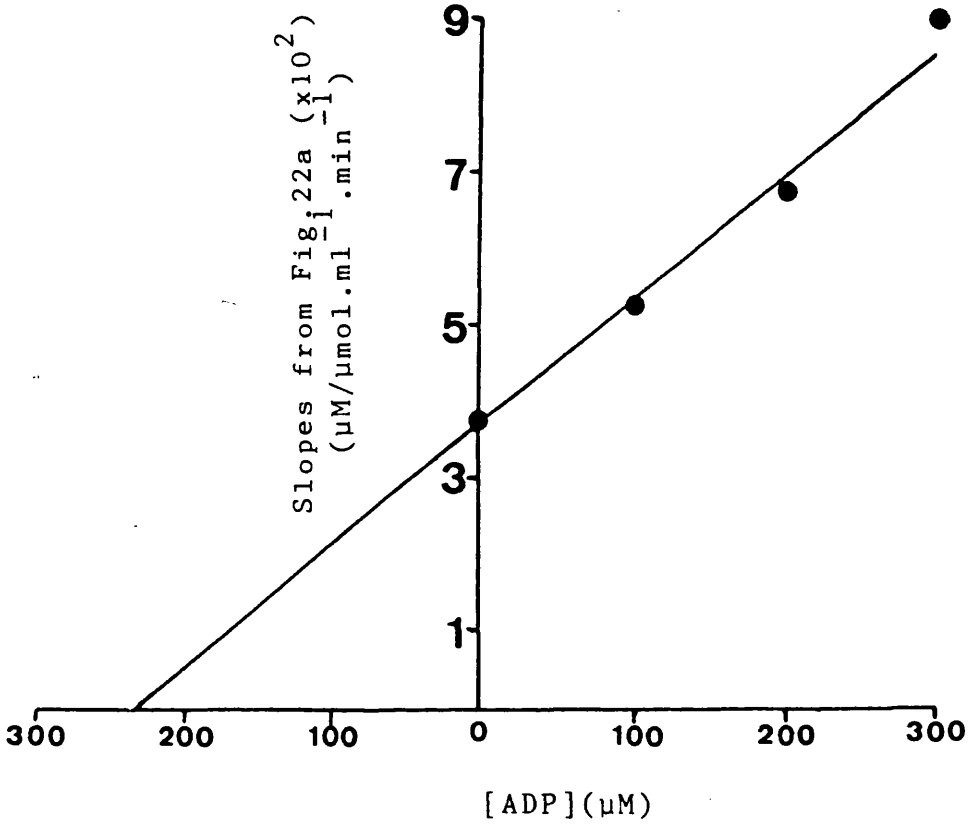


Figure 22b Determination of Ki for ADP of G-STK, using data from Fig. 22a



3·3 Characterization of animal STKs

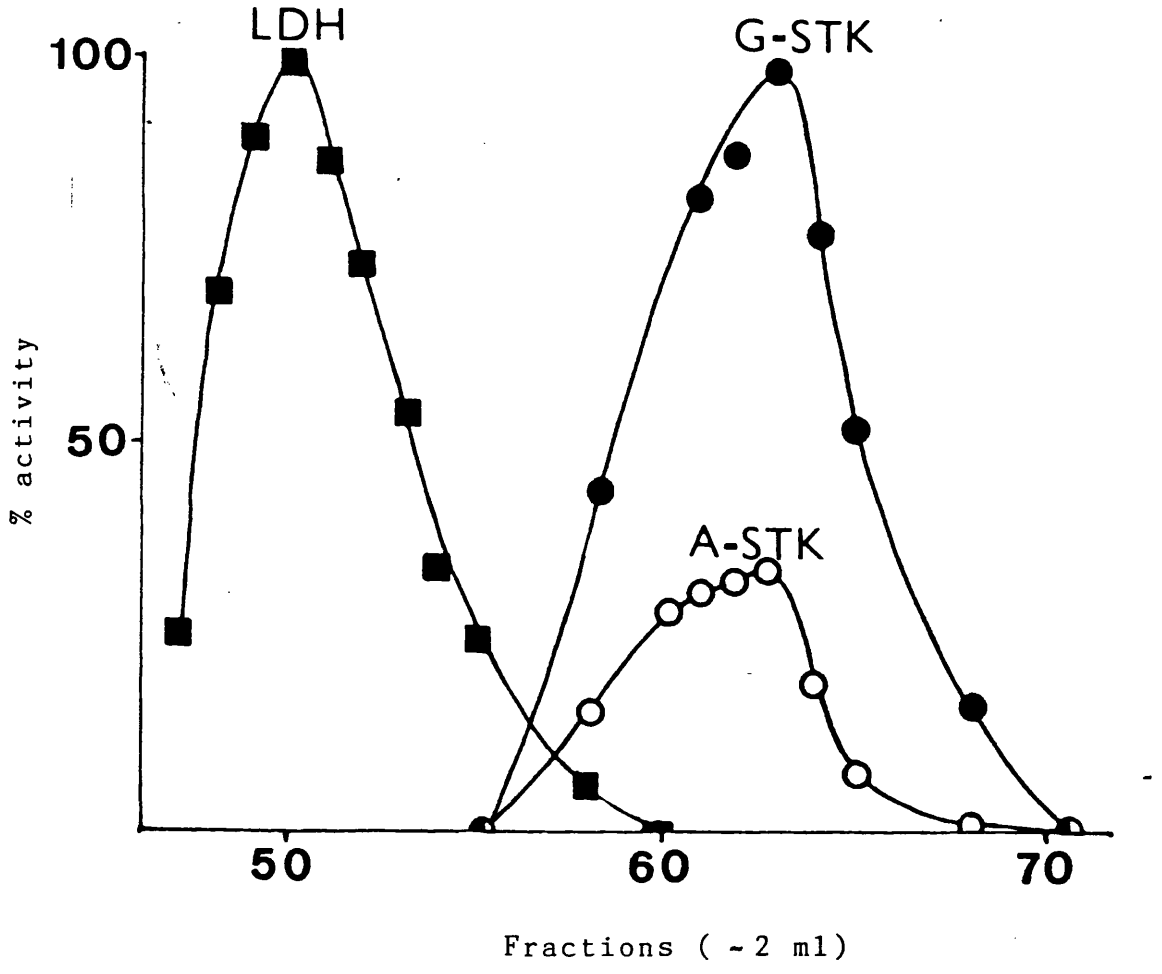
3·3·1 Molecular size

The mammalian G-STK is known to be an αβ dimer of molecular weight around 75,000 (Bridger, 1974). A comparison of the G-STK and newly identified A-STK from mammalian tissues was therefore sought. This was achieved with a simple gel filtration procedure, similar to that used by Weitzman & Kinghorn (1978). As described in section 1·3, the latter authors examined a range of prokaryotic and eukaryotic sources. LDH (Mr ~ 140,000) acted as the marker enzyme, with STKs eluting from the Sephadex G-200 column before LDH termed 'large' (Mr > 140,000) and those eluting after LDH termed 'small' (Mr < 140,000). They clearly showed that Gram-negative bacteria possessed 'large' STKs, similar to the *E. coli* STK (an $\alpha_2\beta_2$ tetramer), while Gram-positive bacteria and eukaryotic STKs were 'small', similar to the pig heart G-STK (an αβ dimer). Fig. 23 shows the gel filtration of rat heart STK activities. Both G-STK and A-STK display very similar elution profiles in respect to their elution after LDH, thus indicating their 'small', possibly dimeric, character. Further studies, on bovine heart mitochondrial G-STK and A-STK, using a calibrated FPLC Superose-12 column, yielded an approximate Mr value of 81,000 for both enzymes.

3·3·2 Nucleotide dependence

The substrate dependences of pig heart G-STK are

Figure 23 Gel filtration of rat heart STKs on a Sephacryl S-200 column at 4°C (2·5 x 35 cm)



well established (Nishimura & Grinnell, 1972). It was therefore interesting to compare the NDP and succinyl-CoA dependences of both G-STK and A-STK from the same source. Figs. 24, 25 & 26 present Hanes-Woolf plots (S/V versus S) of GDP, ADP and IDP, respectively, for the STKs from bovine heart mitochondrial extracts. Figs. 27 & 28 show similar plots for succinyl-CoA dependence. Similar studies were undertaken on the dependence of the STKs of locust flight muscle on ADP & GDP (Figs. 29 & 30). All apparent Km & Vmax values ±.S.D. were calculated using a computer programme of the direct linear plot (Eisenthal & Cornish-Bowden, 1974).

Figure 24 Hanes-Woolf plot: [GDP]/V versus [GDP] for G-STK from bovine heart mitochondria

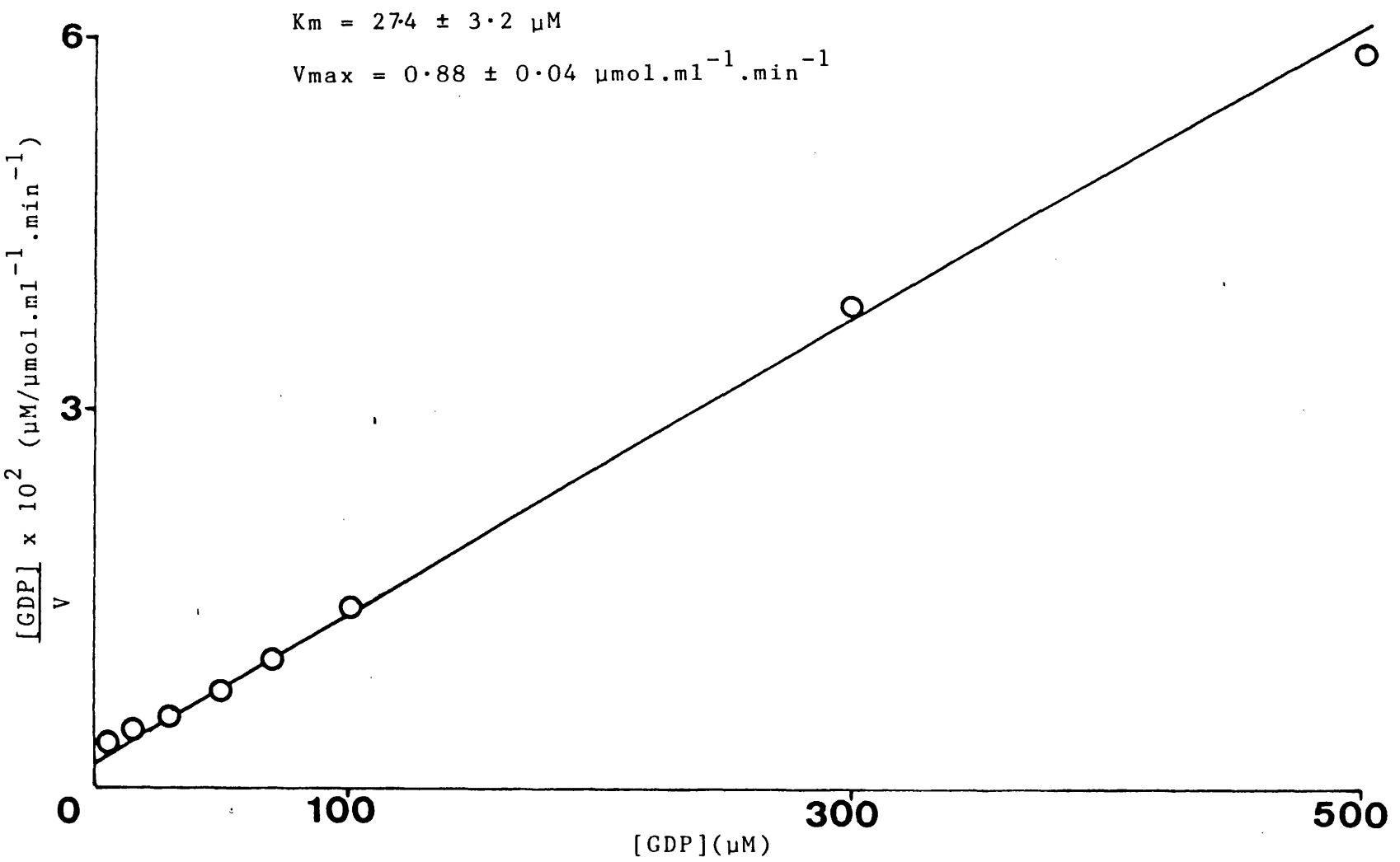


Figure 25 Hanes-Woolf plot: [IDP]/V versus [IDP] for G-STK from bovine heart mitochondria

Km = 42·3 ± 10 µM

Vmax = 0·86 ± 0·1 µmol.ml^{-1}.min^{-1}

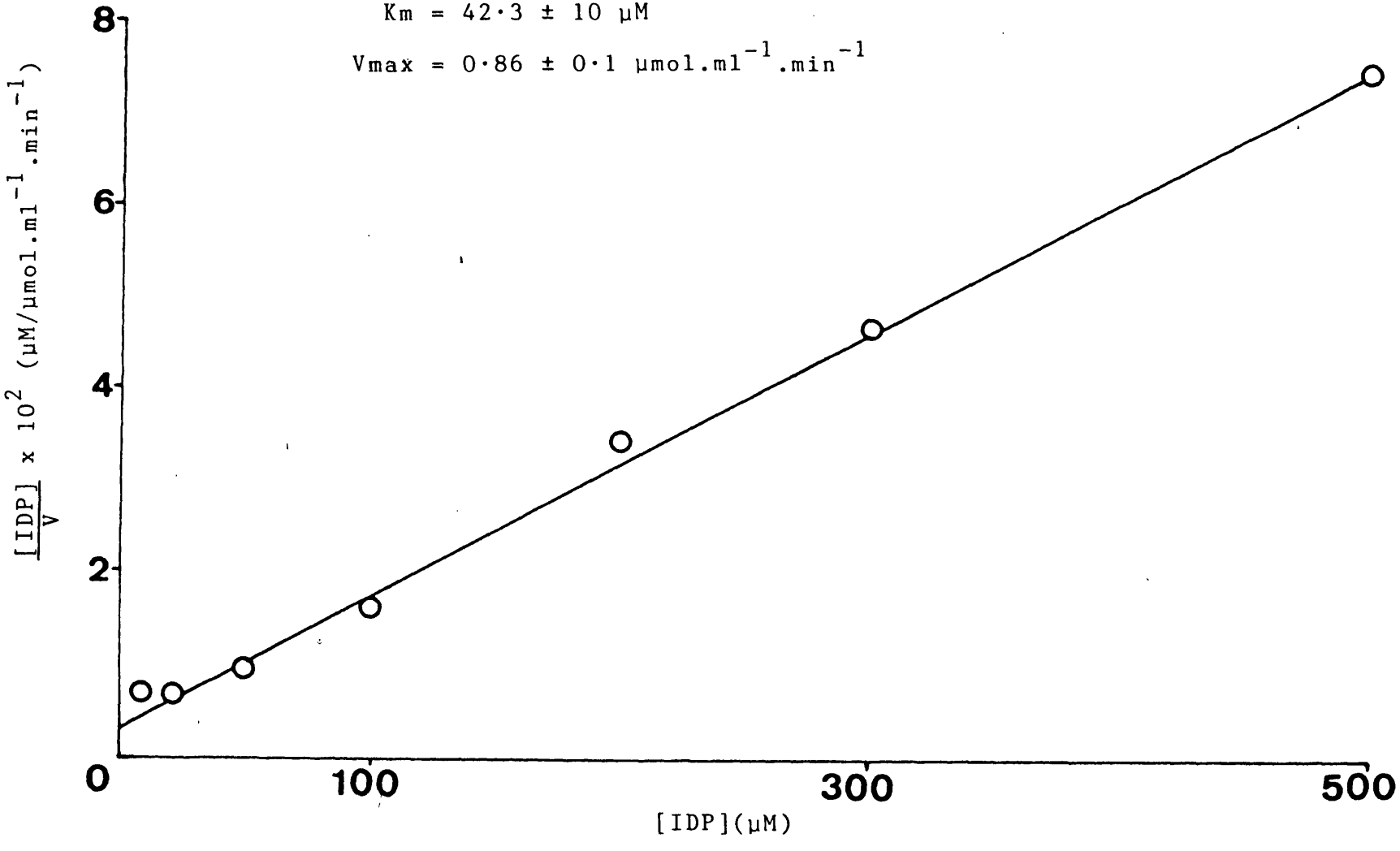


Figure 26 Hanes-Woolf plot: [ADP]/V versus [ADP] for A-STK from bovine heart mitochondria

Km = 57 ± 7 µM

Vmax = 0·49 ± 0·03 µmol.ml^{-1}.min^{-1}

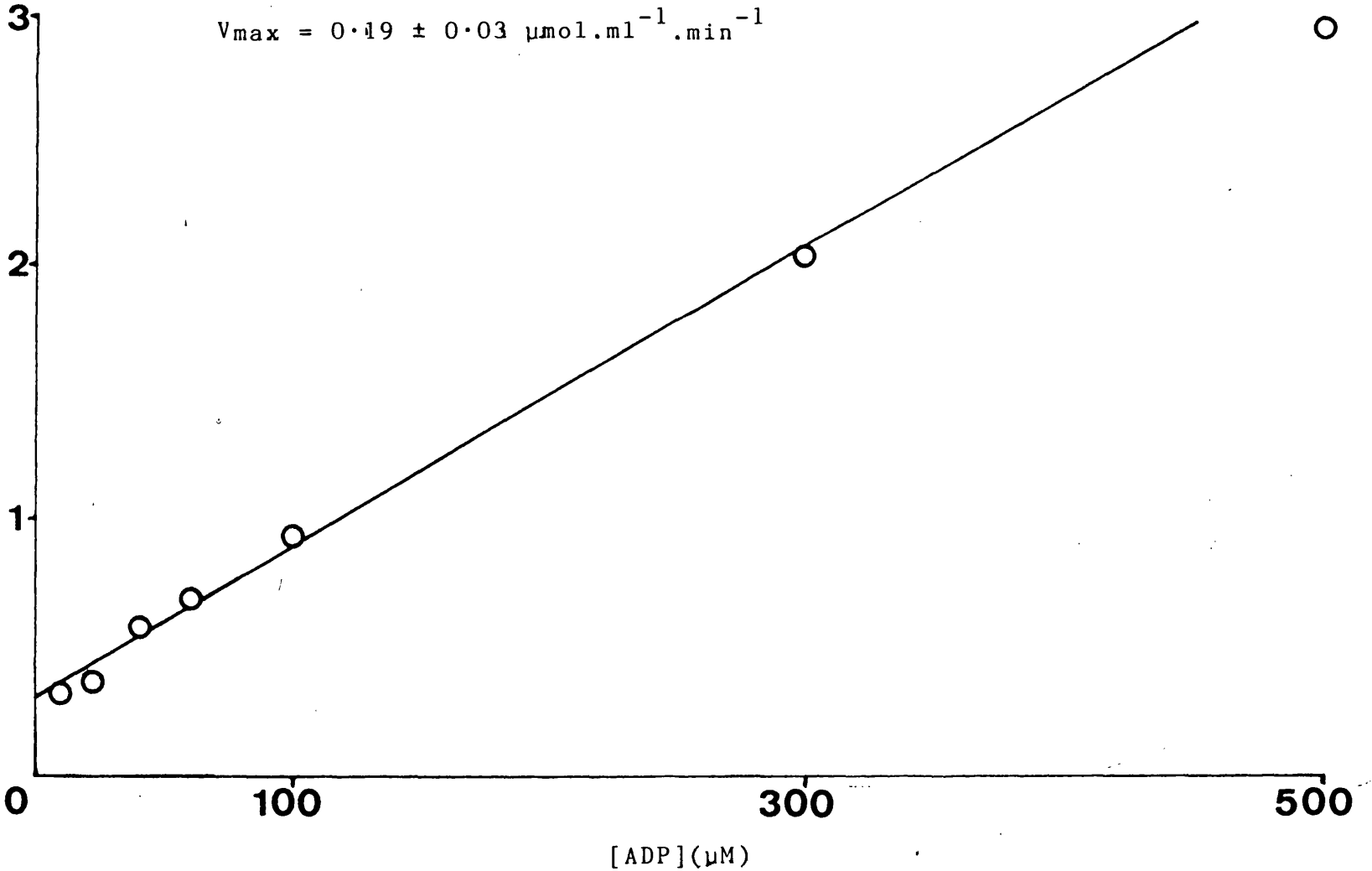


Figure 27 Hanes-Woolf plot: [succinyl-CoA]/V versus [succinyl-CoA] for G-STK from bovine heart

Km = 68·5 ± 20 µM

Vmax = 1·3 ± 0·3 µmol.ml^{-1}.min^{-1}

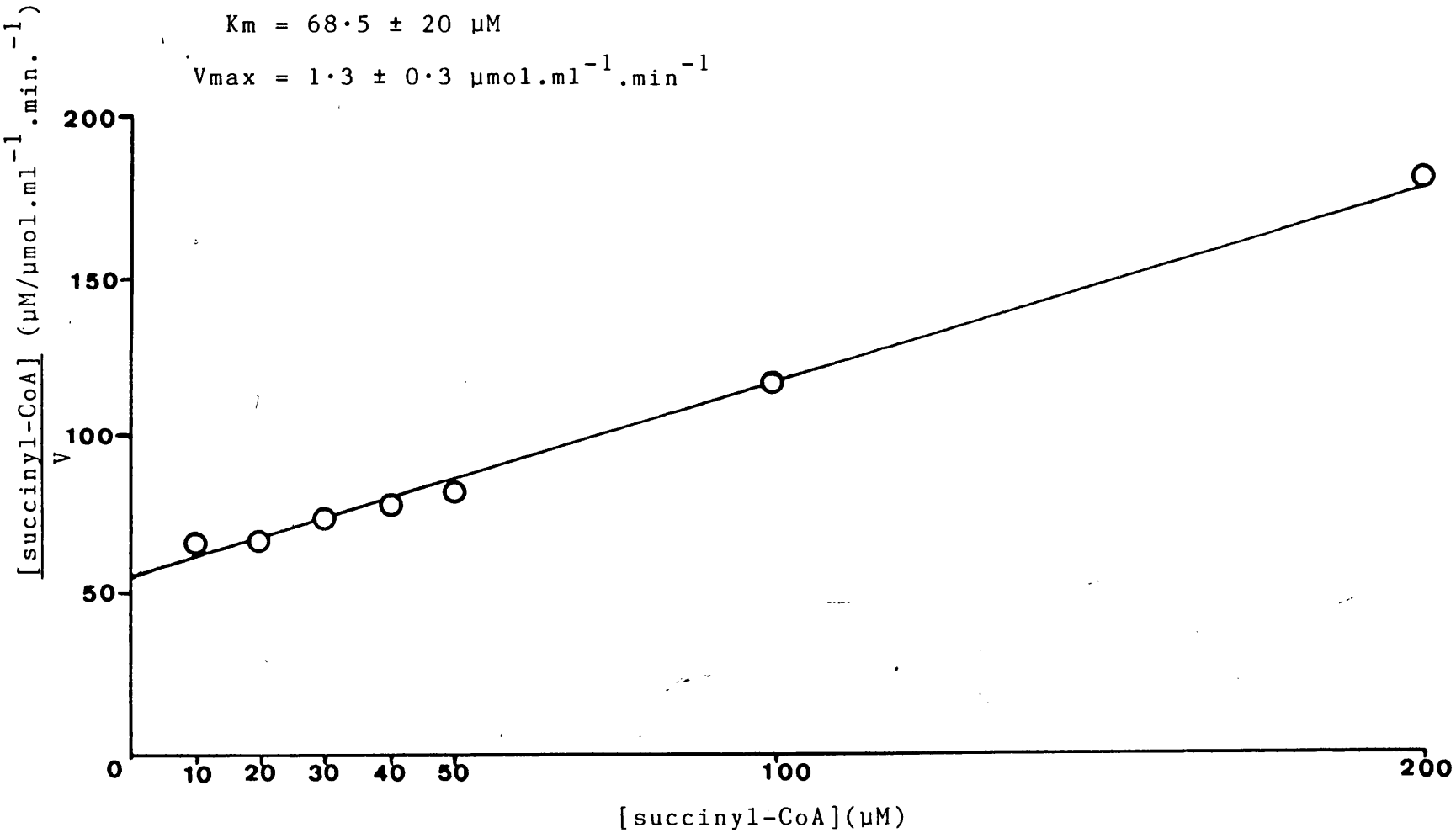


Figure 28 Hanes-Woolf plot: [succinyl-CoA]V versus [succinyl-CoA] for A-STK from bovine heart mitochondria

Km = 48·2 ± 14 μM

Vmax = 0·23 ± 0·05 μmol.ml^{-1}.min^{-1}

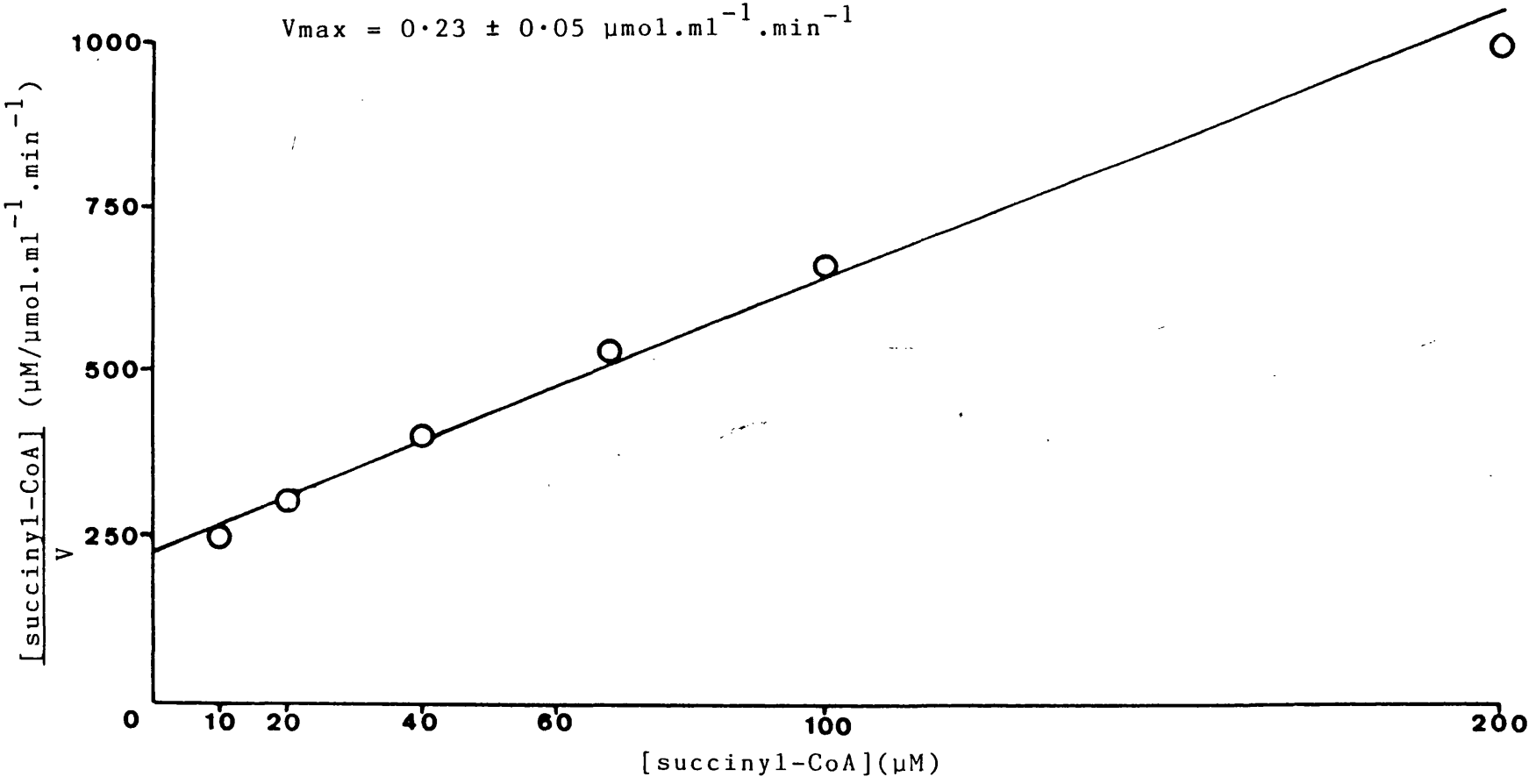


Figure 29 Hanes-Woolf plot: [ADP]/V versus [ADP] for A-STK from locust flight muscle mitochondria

Km = 19·3 ± 4·8 μM

Vmax = 0·57 ± 0·04 μmol.ml^{-1}.min^{-1}

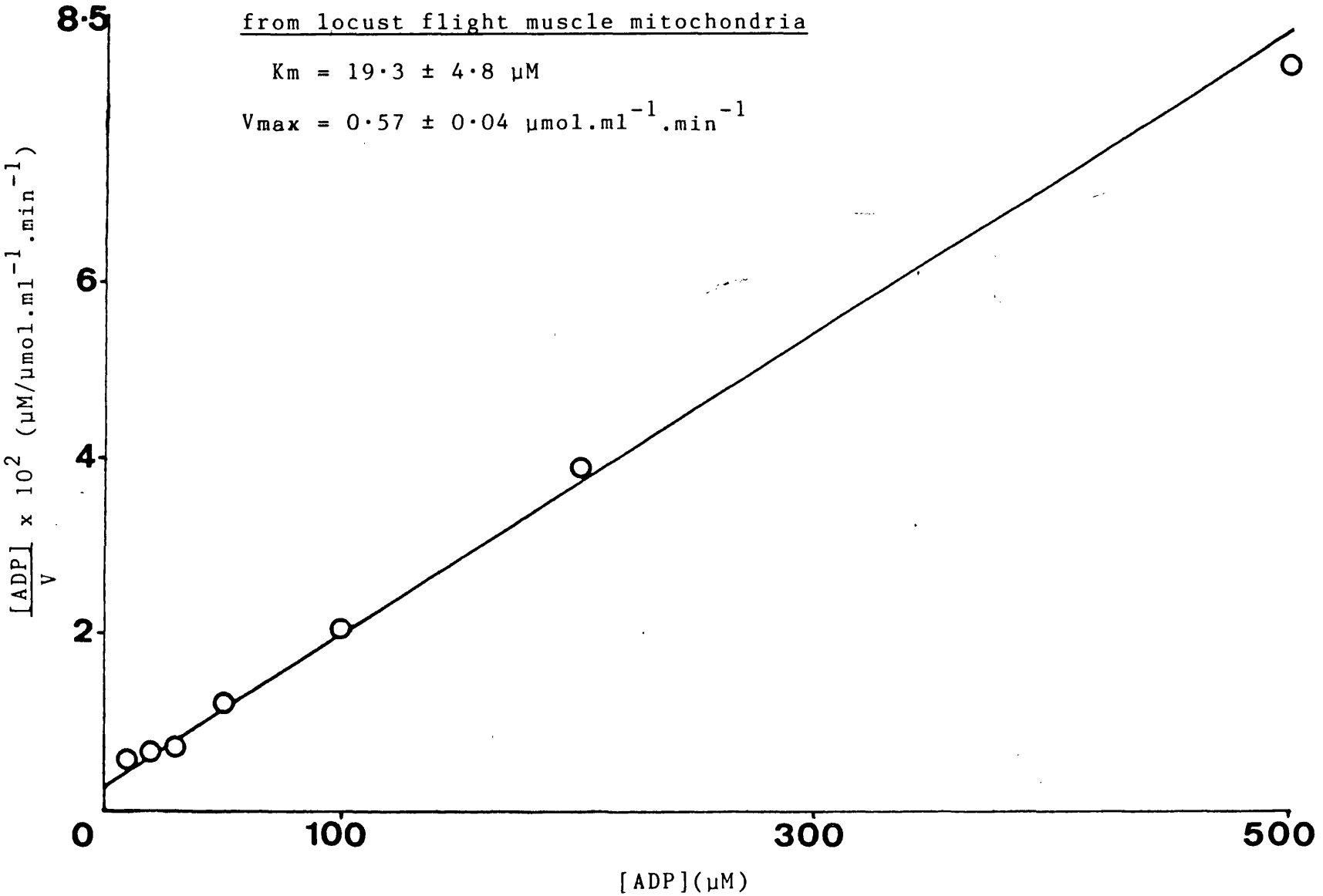
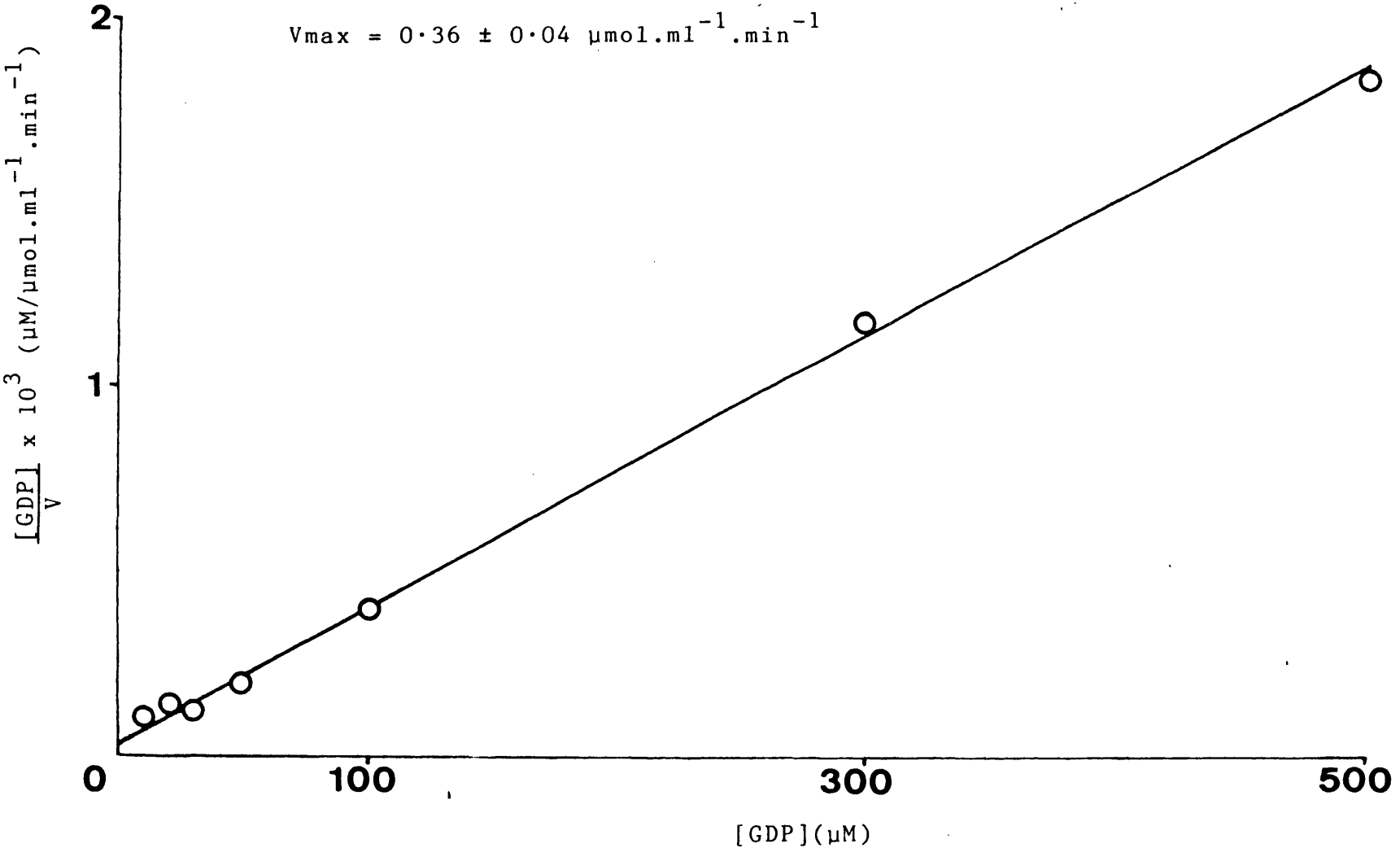


Figure 30 Hanes-Woolf plot: [GDP]/V versus [GDP] for G-STK from locust flight muscle mitochondria

Km = 32·8 ± 8 µM

Vmax = 0·36 ± 0·04 µmol.ml^{-1}.min^{-1}



3·4 STK activities in unicellular eukaryotes

Initially, when *Saccharomyces cerevisiae* (baker's yeast) was examined for STK activity, only A-STK activity could be detected. This result appeared to confirm earlier findings by other investigators (Heerde & Radler, 1978; Schwartz *et al.*, 1983). However, prompted by the results from the mammalian and other animal tissues, permeabilization of yeast cells, instead of the conventional cellular disruption (French press) was carried out. Permeabilization with toluene, of cell or organelle membranes, was first developed by Bridgeland and Jones (1965) and later by Serrano & co-workers (Serrano *et al.*, 1973). It enables small molecular weight substances (e.g. substrates and products) to diffuse freely into and out of the cell or organelle. This technique, unlike the French press which leads to massive dilution of cell constituents, appears to maintain enzymes at their physiological concentrations and probably does not cause extensive disruption of intra-cellular protein-protein interactions. It was previously demonstrated that such *in situ* examinations of enzyme activity in yeast is possible by toluenization (Weitzman & Hewson, 1973). Table 7 presents the results of examining STK activities from *S. cerevisiae* processed by French press or by toluenization. The activities are expressed in $\mu mol.ml^{-1}.min^{-1}.gcell^{-1}$ to enable direct comparison of both procedures. Only A-STK activity was detectable in

French press extracts, whereas both A-STK and G-STK were detected in toluenized cells. It appeared that permeabilization of yeast cells allowed detection for the first time of G-STK activity. It is worth noting that A-STK in permeabilized cells displayed a higher activity compared with that of the French press extract. Also included in Table 7 are the STK activities found in the eukaryotic parasite *Trypanosoma brucei*.

Table 7 STK activities in unicellular eukaryotes

Organism	+ ADP	+ GDP	+ IDP	Ratio
	activity ($\mu mol.ml^{-1}.min^{-1}.g\ cell^{-1}$)			G-STK/A-STK
Saccharomyces cerevisiae				
French press extract	51·4	0	0	-
Toluenized cells	86·0	7·6	7·0	0·09

Trypanosoma brucei	+ ADP	+ GDP	Ratio
	activity ($nmol.min^{-1}.mg^{-1}$)		G-STK/A-STK
Bloodstream form	6·1	1·0	0·16

3·5 STK activities from zea maize

For many years it was assumed that δ-aminolaevulinic acid, required for chlorophyll biosynthesis, originated from succinyl-CoA. This lead to the prediction that either large amounts of mitochondrial succinyl-CoA would have to be transferred to plastids in greening tissues or that plastids contained their own STK activity (Kirk, 1970). The location of STK in photosynthesizing plants was therefore of much interest. Successive attempts, however, failed to find any plastidic STK activity (Fluhr & Harel, 1975). The low levels of apparently chloroplastic STK activities were shown to be entirely due to mitochondrial contamination (Kirk & Pyliotis, 1970).

Beale & Castelfranco (1973) demonstrated that glutamate (C5), rather than succinyl-CoA (C4), was the precursor of chlorophyll. This result removed the necessity for transfer of mitochondrial succinyl-CoA, or a plastidic STK activity.

The discovery of A-STK & G-STK enzymes in other eukaryotic systems prompted a re-examination of STK activities in plant tissues. Table 8 presents the activities of a chloroplastic marker enzyme NADP-phosphoglycerate dehydrogenase (PGADH), together with citrate synthase and STK activities, in etiolated and non-etiolated chloroplasts and mitochondria from zea maize. The first interesting result was that both A-STK and G-STK activities were detected in mitochondria. Citrate synthase and A-STK activities were also found in

Table 8 Enzyme activities from zea maize chloroplast & mitochondrial preparations (etiolated & non-etiolated)

Enzyme	Chloroplast	Mitochondria	Etioplasts	Etioplastic mitochondria
	activity ($nmol.min^{-1}.mg^{-1}$)			
NADP-PGADH	98·6	0	29·6	0
citrate synthase	0	70·6	2·4	10·5
A-STK	0	12·4	4·7	7·5
G-STK	0	1·5	0	0

After extraction, chloroplasts or etioplasts were separated from mitochondria by rapid centrifugation (8,000 x g for 10 s at 4°C). Mitochondria were pelleted at 30,000 x g for 30 min at 4°C.

etioplasts, but not in chloroplasts. The finding of both STK activities in plant mitochondria, mirrors the results obtained with other eukaryotic systems. The fact that no STK activity could be found in chloroplasts, complements previous studies and is consistent with glutamate, not succinyl-CoA, acting as the chlorophyll precursor. The surprising finding of citrate synthase and A-STK activity in etioplasts is less easily explained. If it is due to contamination with mitochondria, then similar contamination would be expected to occur in the chloroplastic fraction. In fact, etioplast contamination of mitochondria would be far more likely to occur because etioplasts are known to be considerably smaller than chloroplasts (Kirk & Tilney-Bassett, 1978). The metabolism of the etioplast is not well established and it may be that during etioplast development, respiration is occurring within it.

3·6 Purification of pig liver G-STK and pigeon breast muscle A-STK

G-STK enzymes have previously been purified from mammalian heart & liver tissues (Cha & Parks, 1964; Ball & Nishimura, 1980), but no A-STK enzyme has been purified from any animal source since Hansford (1973) first identified an A-STK in blowfly flight muscle. After our discovery of distinct STKs in animal tissues, each possessing strict nucleotide specificity, it became apparent that purification of these enzymes from a single source may yield information concerning subunit function. The purified enzymes would be dissociated into their respective α and β subunits and an attempt would be made to construct catalytically active hybrid (αβ) molecules, by recombination of heterologous α and β subunits. The procedure of dissociation and recombination of modified subunits of the *E.coli* tetrameric ($\alpha_2\beta_2$) enzyme has already successfully been demonstrated (Pearson & Bridger, 1975a). Such a procedure applied to the eukaryotic system may determine the subunit governing nucleotide recognition. It is possible that one subunit is conserved between the two dimeric enzymes, e.g. the β subunit may be common to both STKs, as it appears to possess the succinate and CoA binding sites, whereas the α subunit, which is phosphorylated during catalysis, may vary, contributing the strict nucleotide specificity of each STK. Before the purification of G-STK and A-STK enzymes from the same source was undertaken, it was proposed that purification

procedures should be established using convenient sources of each enzyme. Pig liver (G-STK) and pigeon breast muscle (A-STK) were chosen as rich sources of each enzyme.

3·6·1 Purification procedures for both enzymes

Both purifications followed similar procedures up to, and including, affinity chromatography (Tables 9 & 10). All procedures were carried out at 4°C, except for the affinity and fast protein liquid chromatographic steps which were done at room temperature.

Each tissue was homogenized with a Waring blender in extraction buffer (0·1 M sodium/potassium phosphate buffer, pH 7·5, containing 1 mM EDTA, 0·1 mM EGTA and 25 mM ascorbate). After centrifugation (30,000 x g for 30 min), $(NH_4)_2SO_4$ fractionation of the supernatants and dialysis (2 x 2 h against 2 1 of 10 mM sodium/potassium phosphate buffer, pH 7·5, containing 1 mM EDTA, 25 mM ascorbate and 0·5% (v/v) glycerol) the samples were loaded onto hydroxylapatite columns, equilibrated with 30 mM Tris-acetate buffer, pH 8·0, containing 1 mM EDTA, 25 mM ascorbate and 0·5% (v/v) glycerol. After washing with one column volume of the Tris-acetate buffer, linear phosphate gradients were established (10 mM-350 mM potassium phosphate buffer, pH 7·5, containing 1 mM EDTA, 25 mM ascorbate and 0·5% (v/v) glycerol) and ~ 3 ml fractions were collected overnight. Pig liver G-STK was eluted at ~ 250 mM phosphate, whereas pigeon breast A-STK was eluted at ~ 200 mM phosphate. Fractions with the

Table 9 Purification of pig liver G-STK

Sample	Volume (ml)	Total protein (mg)	Total activity ($\mu mol.ml^{-1}.min^{-1}$)	Specific activity ($\mu mol.min^{-1}mg^{-1}$)	Purification (-fold)	Yiel (%)
Crude	192	8830	117·8	0·01	-	100
$(NH_4)_2SO_4$ 40-55%	30	1690	38·2	0·023	1·77	32
hydroxylapatite column	25	105	32·9	0·38	29·0	28
dial-GDP affinity column	2	0·95	7·9	8·32	639	6·7

0·40 % $(NH_4)SO_4$ = 0·243 g/ml at 4°C

40-60% " = 0·C97 g/ml at 4°C.

Flow rate of hydroxylapatite and affinity columns = 12 ml h^{-1}

Table 10 Purification of pigeon breast A-STK

Sample	Volume (ml)	Total protein (mg)	Total activity ($\mu mol.ml^{-1}.min^{-1}$)	Specific activity ($\mu mol.min^{-1}.mg^{-1}$)	Purification (-fold)	Yiel (%)
Crude	210	7350	20·6	0·0028	-	100
$(NH_4)_2SO_4$ 40-60%	13·5	945	12·5	0·0132	4·7	60·7
hydroxylapatite column	8·5	59·5	6·8	0·114	40·7	33
dial-ADP affinity column	2	4·6	4·2	0·913	326	20·4
FPLC (monoQ)	2	1·2	2·2	1·833	655	10·6

0-40 % $(NH_4)_2SO_4$ = 0·243 g/ml at 4°C

40-60 % " = 0·132 g/ml at 4°C

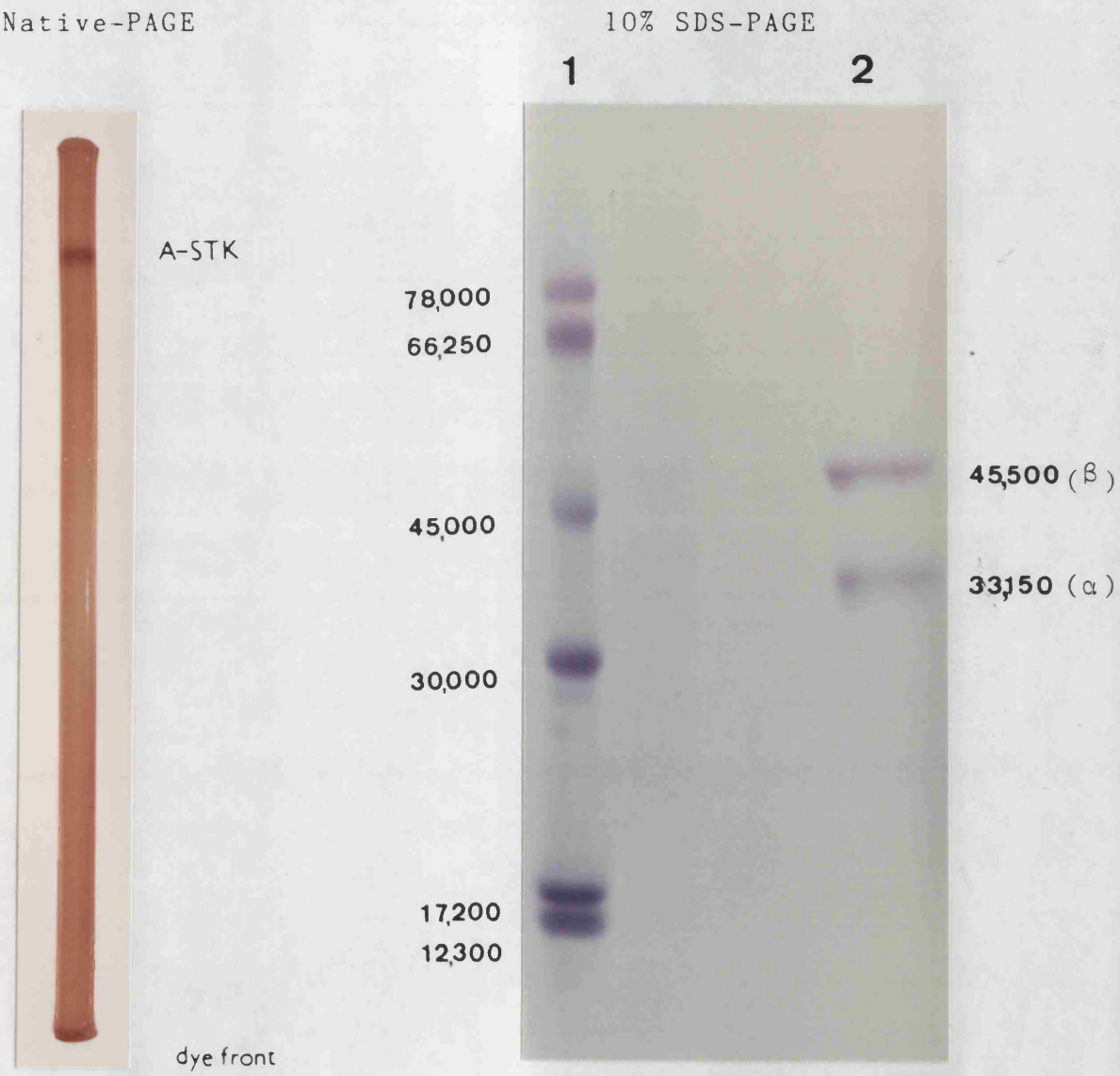
Flow rate of hydroxylapatite and affinity columns = 12 ml h^{-1}

Flow rate of FPLC column = 1 ml min^{-1}.

highest specific activity were pooled and concentrated using an Amicon ultrafiltration concentrator and subsequently stored at -80°C overnight. Concentrated fractions were desalted using prepacked PD-10 columns into 25 mM Tris-HCl buffer, pH 7·5, containing 2 mM $MgCl_2$ and 25 mM ascorbate. Pig liver and pigeon breast samples were loaded onto their respective dial 3',5'-NDP-adipic acid hydrazide Sepharose 4B columns, which were then washed until no more protein was eluted. The STKs were then eluted with their nucleotide buffer - 20 mM sodium/potassium phosphate buffer, pH 7·5, containing 2 mM $MgCl_2$ and 2 mM GDP or ADP. Fractions apparently containing STK activity were then desalted on PD-10 columns to remove the nucleotide buffer. The pigeon A-STK was then loaded, in 20 mM Tris-HCl buffer, pH 7·5, containing 2 mM $MgCl_2$, onto a FPLC Mono Q column (see section 2·15·6) and eluted with a 0-200 mM KCl gradient (Fig. 17). All attempts to bind pig G-STK to Mono Q (anion exchanger) or Mono S (cation exchanger) proved unsuccessful, with no increase in specific activity.

Both enzymes were shown to be electrophoretically homogeneous by polyacrylamide gel electrophoresis (PAGE) under non-denaturing conditions, with silver or Coomassie brilliant blue staining. Subunit molecular weight determinations were achieved on SDS-PAGE gels using standard molecular weight proteins. Pigeon breast A-STK gave subunit Mr of 33,150 (α subunit) and 45,000 (β subunit) (Figs. 31 & 32), while pig liver G-STK gave Mr of

Figure 31 Photographs of native and SDS-PAGE of purified pigeon breast A-STK



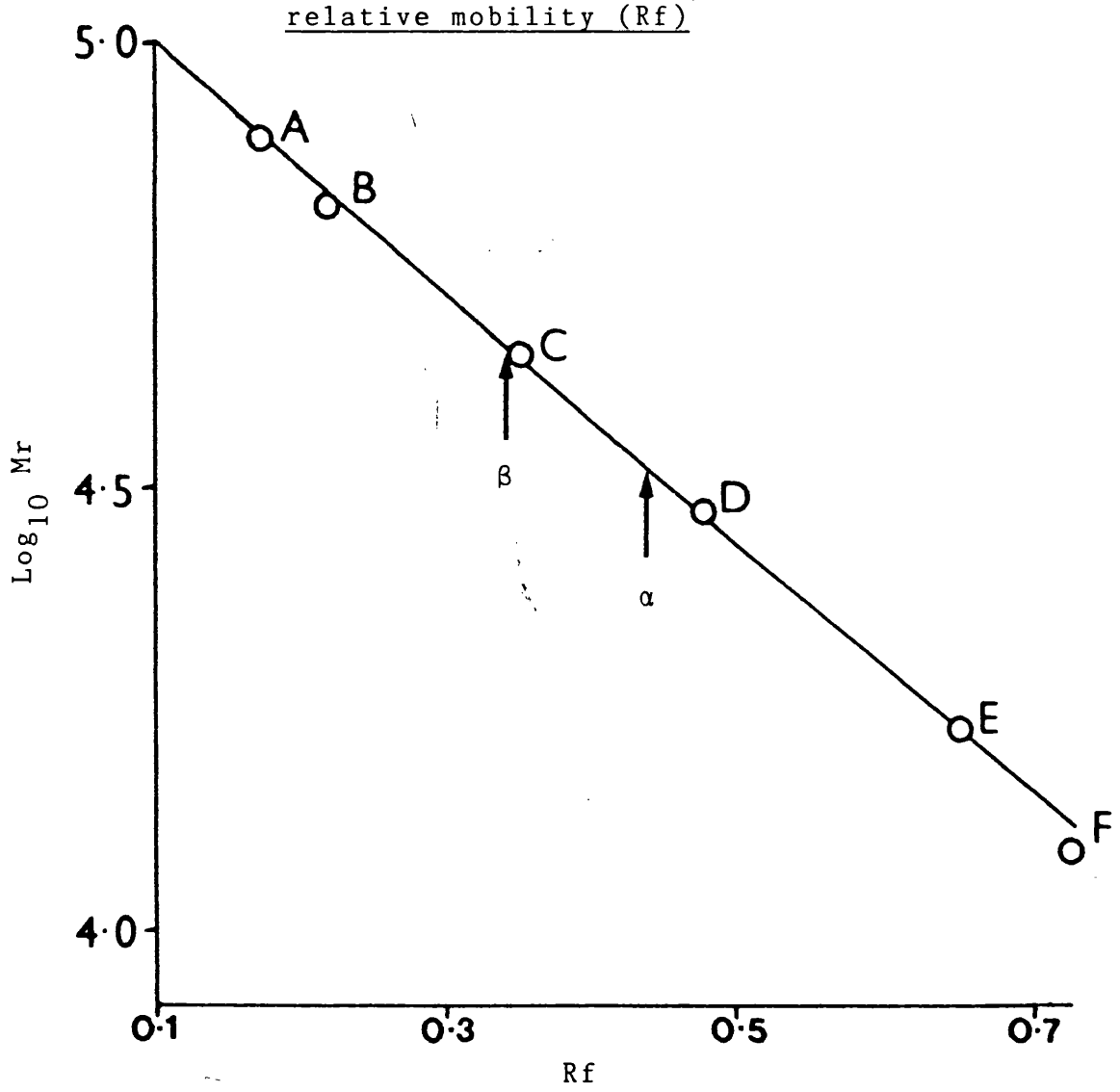
5 µg purified pigeon breast A-STK loaded; protein was detected by silver staining.

Track 1 - standard Mr subunits (10 µg each);
Track 2 - purified pigeon breast A-STK (15 µg); protein was detected by Coomassie brilliant blue staining.

Figure 32

Standard Mr subunits: (A), hen egg - Ovotransferrin (Mr 78,000); (B), bovine serum - Albumin (Mr 66,250); (C), hen egg - Ovalbumin (Mr 45,000); (D), bovine erythrocyte - Carbonic anhydrase (Mr 30,000); (E), equine - Myoglobin (Mr 17,200) and (F), equine - Cytochrome c (Mr 12,300). Pigeon breast muscle A-STK dissociated in SDS to yield an α-subunit (Mr 33,150) and a β subunit (Mr 45,500).

Figure 32 SDS-polyacrylamide gel electrophoresis of pigeon breast muscle A-STK, with standard Mr proteins, at pH 8·9 - Relationship between $\log_{10}$ Mr and relative mobility (Rf)



34,100 (α subunit) and 46,300 (β subunit) (data not shown). Brownie & Bridger (1972) have already characterized an animal G-STK (pig heart) and shown it to be a dimer with an α subunit (Mr = 34,500) and β subunit (Mr = 42,500). Ball & Nishimura (1980) obtained very similar subunit molecular weights for rat liver G-STK (Mr α = 33,500 & Mr β = 46,500). Confirmation of the dimeric nature of both the pig liver G-STK and pigeon breast A-STK, was obtained by using a calibrated FPLC Superose-12 column (Fig. 33).

During the establishment of a purification procedure for pigeon breast A-STK, Allan & Ottaway (1986) published information concerning a partially purified A-STK fraction from pigeon breast muscle. They obtained a native enzyme molecular weight of ~ 100,000 by gel filtration and subunit molecular weights of ~ 40,000 (α) and ~ 48,000 (β) by SDS-PAGE. These values are slightly higher than those obtained from Figs. 32 & 33 for the purified pigeon breast A-STK, and from the other animal STKs so far investigated.

3·6·2 Characterization of pigeon breast A-STK

As mentioned above, Allen & Ottaway (1986) recently published molecular and kinetic information on partially purified pigeon breast A-STK. They obtained a Km for CoA of approx. 20 μM, which is comparable with the value reported for pig heart G-STK (Cha, 1969). However, from a Lineweaver-Burk plot, they obtained a Km for ATP of 0·8 mM. Yet on examination of their published data, the

Figure 33

Determination Kd: $Kd = (Ve - Vx)/(Vy - Vx)$

where Ve = elution vol. of protein

Vx = exclusion vol. of Blue Dextran 2000 ($Mr \sim 3 \times 10^6$)

Vy = elution vol. of dnp-lysine (Mr = 367)

Exclusion vol. = 6·0 ml

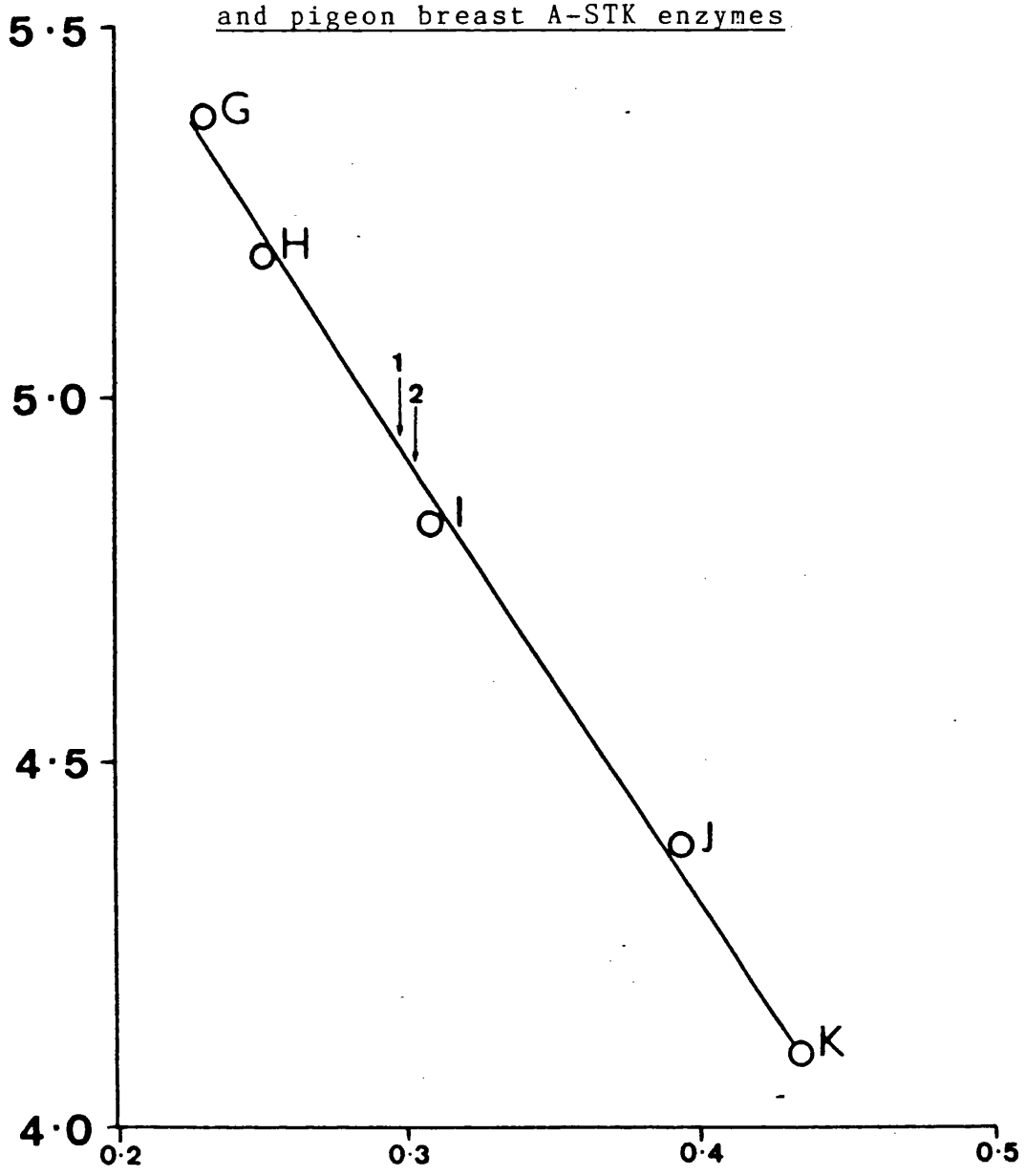
Inclusion vol. = 29·8 ml

Standard proteins: (G), bovine liver - catalase (Mr 240,000); (H), rabbit muscle - aldolase (Mr 158,000); (I), bovine serum - albumin (Mr 68,000); (J), bovine pancreas - chymotrypsinogen (Mr 25,000); (K), equine heart - cytochrome c (Mr 12,500).

1 - Pig liver G-STK Mr 85,100

2 - Pigeon breast A-STK Mr 79,400

Figure 33 FPLC gel filtration (Superose-12 column)
Mr determination of purified pig liver G-STK
and pigeon breast A-STK enzymes

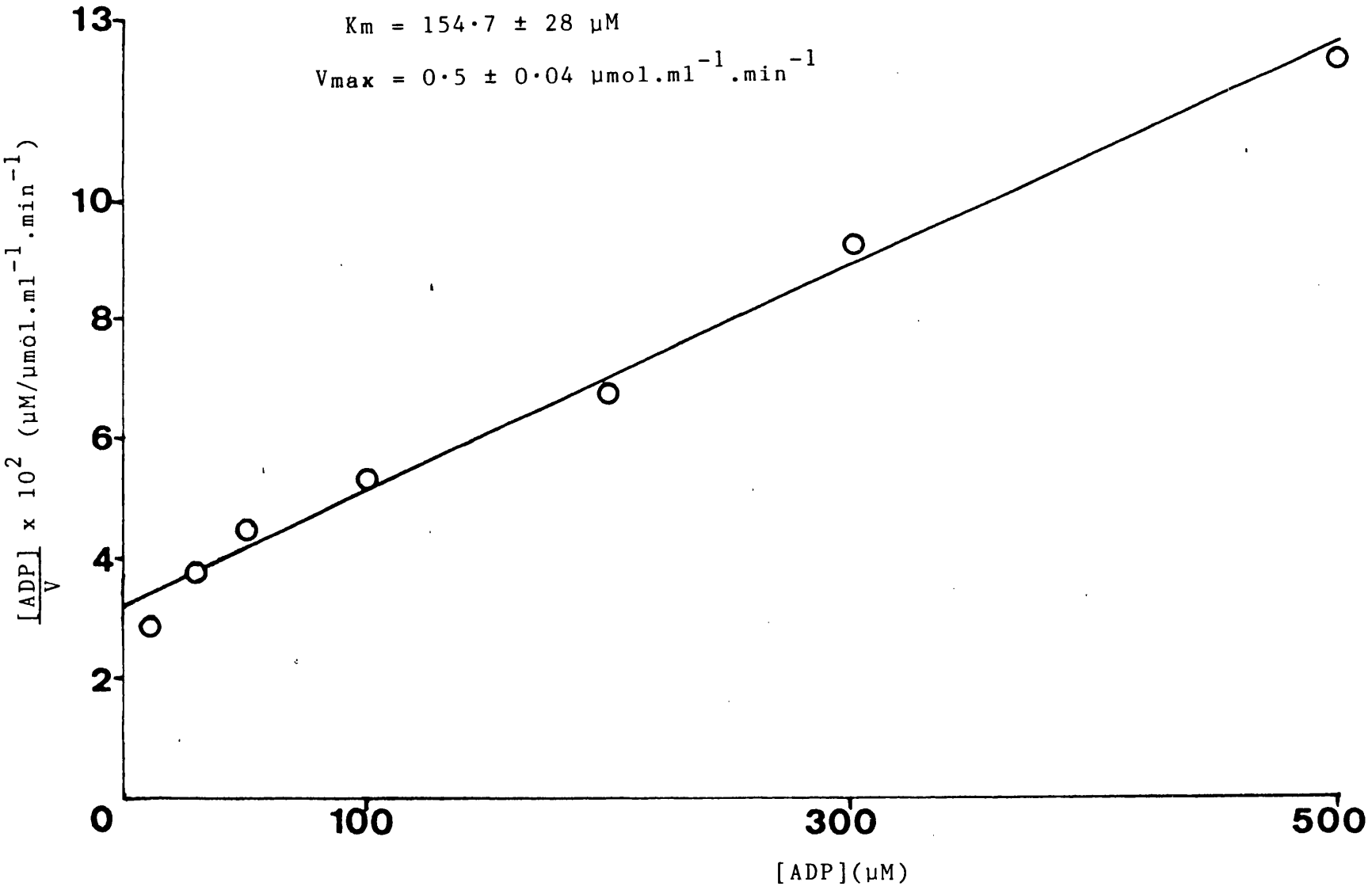


Standard Mr proteins (0·5 mg each) loaded in 200 µl on to column equilibrated with 0·1 M Tris-HCl + 20% glycerol buffer, pH 8·0. 200 µl fractions collected. Flow rate = 0·3 ml/min.

intercept on the abscissa yields a Km value of ~ 1·6 mM! In contrast, Km values for GTP from mammalian G-STK enzymes are of the order of 10-30 μM (Cha, 1969; Ball & Nishimura, 1980). Hansford (1973), in studies of A-STK from blowfly flight muscle, obtained Km values of 0·29 mM for both ATP and ADP. The present studies on the purified pigeon breast A-STK yielded a Km value of 155 ± 28 μM for ADP (Fig. 34), which is similar to the results of Hansford (1973). However, these Km values for ADP with the pigeon breast and blowfly A-STKs are substantially higher than those for bovine heart and locust flight muscle A-STKs. Here, Km values for ADP are of the order of 20-40 μM, more nearly matching the Km values for guanine nucleotides with the animal G-STKs.

Although Allen & Ottaway (1986) could not detect G-STK activity in pigeon breast muscle, the present study consistently detected G-STK activity in the $(NH_4)_2SO_4$ 40-60% fractions during the purification of A-STK (Table 4). Purification of a G-STK from pigeon breast muscle has been reported (Meshkova & Matveeva, 1970). However, such a discrepancy has been commented upon (Allen & Ottaway, 1986) and from the above information cannot be explained.

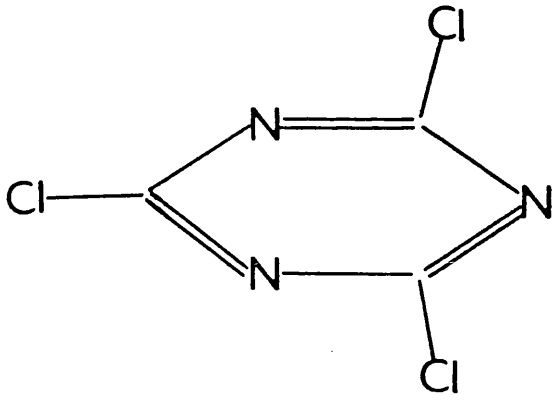
Figure 34 Hanes-Woolf plot: [ADP]/v versus [ADP] for A-STK from pigeon breast muscle



3·7 Preliminary purification of A-STK and G-STK from a single source

From examining a wide range of animal tissues it became apparent that the best source of both STKs was bovine heart. Unfortunately, initial purification attempts using the procedures previously described were unsuccessful, especially in the case of A-STK, which displayed instability. Attempts to stabilize the enzyme over the 3-4 day procedure with protease inhibitors, glycerol and reducing agents were only partially successful. It was therefore concluded that an additional rapid separation procedure was required. Dye-ligand Chromatography was chosen as a possible further purification step.

Dye-ligand chromatography developed after it was noticed that occasionally, during gel filtration, certain proteins co-eluted with the void volume marker dextran blue (a dextran conjugate of cibacron blue-F3GA). It was soon established that these proteins were binding selectively to the dye (Kopperschlager *et al.*, 1968). After these initial observations the possibility of triazine dye chromatography was studied and shown to be effective for a wide range of proteins (Dean & Watson, 1979) and especially nucleotide-requiring enzymes (Turner, 1981). Triazine dyes were originally developed by ICI Chemicals, primarily for use in the textile industry. They are based on the chemistry of cyanuric chloride (1,3,5,-trichlorotriazine).



1,3,5, trichlorotriazine, the basic element of triazine dyes

The procion range of dyes, which contain the reactive chlorotriazine moiety, consists of various aromatic chromophores linked to the triazine ring via NH-bridges. Procion MX dyes contain one substitution, whereas the Procion HE range contain two substitutions to the triazine ring. The dyes are readily coupled, by direct substitution of the chlorine atom(s) in the ring, with hydroxyl groups on the supporting matrix.

For successful binding of a protein/enzyme the pH, temperature and ionic strengths are important variables. Elution can be achieved either by pH/ionic gradient elution or by bio-specific elution. Bio-specific elution utilizes the affinity of an enzyme for its substrate(s), product(s) or substrate(s) and product(s) to produce a dead-end complex.

Preliminary studies for identification of a suitable triazine dye column, for both A-STK and G-STK, are shown in Tables 11 and 12, respectively. (The dye identification list occurs in section 2·15·4). The 5 ml

Table 11 Preliminary screening of triazine dye columns for G-STK from bovine heart

Column No	Protein buffer wash (mg/ml)	Total activity ($\mu mol.min^{-1}$)	Protein 1 M KCl wash (mg/ml)	Total activity ($\mu mol.min^{-1}$)	Specific activity ($nmol.min^{-1}.mg^{-1}$)	% Recovery
1	1·5	0	1·65	0·23	27·8	46
2	0·5	0	2·5	0·22	17·8	44
3	2·13	0·037	1·3	0·27	41·5	54
4	1·1	0	2·7	0·14	10·5	28
5	1·4	0·027	2·35	0·27	23·0	54
6	1·5	0	2·2	0·23	21·2	46
7	0·95	0	3·1	0·21	13·7	42
8	0·83	0	3·0	0·15	10·1	30
9	0·5	0	2·1*	0·17*	50·35*	34*
10	0·8	0·017	2·35	0·13	11·5	26
11	1·95	0	2·1	0·26	24·7	52
12	2·2	0·088	1·65	0·14	16·8	28
13	0·75	0	2·7	0·08	6·2	16
14	3·6	0	0·95	0·31	64·6	62
15	1·3	0	2·7	0·21	15·2	42

(0·5 units loaded per column, with a specific activity of 18·5 $nmol.min^{-1}.mg^{-1}$).

5 ml buffer wash and 5 ml salt wash fractions collected.

*column 9 - G-STK required 2 M KCl for elution.

Table 12 Preliminary screening of triazine dye columns for A-STK from bovine heart

Column No	Protein buffer wash (mg/ml)	Total activity (μmol.min^{-1})	Protein 1 M KCl wash (mg/ml)	Total activity (μmol.min^{-1})	Specific activity (nmol.min^{-1}.mg^{-1})	% Recovery
1	1·5	0	1·65	0·043	5·2	43
2	0·5	0	2·5	0·051	4·0	51
3	2·13	0	1·3	0·051	7·8	51
4	1·1	0·017	2·7	0·051	3·7	51
5	1·4	0	2·35	0	0	0
6	1·5	0	2·2	0·057	5·2	57
7	0·95	0	3·1	0·02	1·3	20
8	0·83	0·02	3·0	0·037	2·5	37
9	0·5	0·034	2·1	0	0	0
10	0·8	0·034	2·35	0·034	2·9	34
11	1·95	0·03	2·1	0·027	2·6	27
12	2·2	0	1·65	0	0	0
13	0·75	0	2·7	0·02	1·5	20
14	3·6	0	0·95	0·064	13·5	64
15	1·3	0	2·7	0	0	0

(0·1 units loaded per column, with a specific activity of 3·7 nmoles min^{-1}mg^{-1}).

5 ml buffer wash and 5 ml salt wash fractions were collected.

(bed volume) columns were equilibrated at 4°C with 20 mM Tris-HCl buffer, pH 7·5, containing 0·5 mM EDTA, 2 mM $MgCl_2$ and 25 mM ascorbate. After loading samples (desalted $(NH_4)_2SO_4$ 40-65% fractions of bovine heart crude tissue extracts) and washing with equilibrating buffer, the columns were eluted with 1 M KCl in equilibrating buffer. Both G-STK and A-STK bound to all columns to various extents. However, for both enzymes, column 14 (yellow MXG8) yielded the greatest increase in specific activity and the highest percentage recovery. Earlier work in this laboratory had demonstrated that a range of citrate synthase and STK enzymes were able to bind to procion Red HE3B (column 2) and then be eluted with KCl (Weitzman & Ridley, 1983). The results displayed in Table 11 for bovine heart G-STK on column 2 were comparable, with respect to percentage recovery, with the result for pig liver G-STK in that earlier study.

After identification of yellow MXG8 as a suitable dye ligand, biospecific elution was attempted. Tables 13 & 14 present the results for G-STK and A-STK respectively. The eluants for each column were as follows: 1 - 0·2 mM succinyl-CoA; 2 - 2 mM succinate; 3 - 0·2 mM CoA; 4 - 1 mM GTP; 5 - 1 mM GDP; 6 - 1 mM ATP; 7 - 1 mM ADP; 8 - 2 mM succinate + 1 mM GTP; 9 - 2 mM succinate + 1 mM ATP; 10 - 0·2 mM succinyl-CoA + 0·5 mM potassium phosphate; 11 - 0·2 mM succinyl-CoA + 1 mM GTP; 12 - 0·2 mM succinyl-CoA + 1 mM ATP; 13 - 0·2 mM CoA + 2 mM succinate + 1 mM GDP; 14 - 0·2 mM CoA + 2 mM succinate + 1 mM ADP; 15 - 0·1 M KCl.

Table 13 Bio-specific elution of G-STK (bovine heart) from triazine dye column yellow MXG8

Column No.	Protein (mg/ml)	Total activity (μmol.min^{-1})	Specific activity (nmol.min^{-1}.mg^{-1})	% Recovery
1	0·11	0·41	375	82
2	0·05	0·14	270	28
3	0·15	0·31	207	62
4	0·09	0·2	215	40
5	0·06	0·15	245	30
6	0·09	0·175	199	35
7	0·066	0·136	206	27
8	0·09	0·27	207	54
9	0·11	0·069	63	14
10	0·09	0·175	77	35
11	0·09	0·35	383	70
12	0·09	0·337	199	67
13	0·19	0·37	195	74
14	0·15	0·365	243	73
15	0·15	0·243	162	47

0·5 units loaded per column, with a specific activity of 16·1 nmol.min^{-1}.mg^{-1}.

10 ml buffer wash & bio-specific wash fractions were collected.

Table 14 Bio-specific elution of A-STK (bovine heart) from triazine dye column yellow MXG8

Column No.	Protein (mg/ml)	Total activity ($\mu mol.min^{-1}$)	Specific activity ($nmol.min^{-1}.mg^{-1}$)	% Recovery
1	0·11	0·24	218	80
2	0·05	0·10	200	33
3	0·15	0·135	90	45
4	0·09	0·175	195	58
5	0·06	0·095	157	31
6	0·09	0·13	140	43
7	0·066	0·148	225	50
8	0·09	0·121	135	40
9	0·11	0·162	147	54
10	0·09	0·108	120	36
11	0·09	0·135	150	45
12	0·09	0·162	181	54
13	0·19	0·16	84	53
14	0·15	0·24	162	80
15	0·15	0·094	63	31

0·3 units loaded per column, with a specific activity of 5·3 $nmol.min^{-1}mg^{-1}$.

10 ml buffer wash & bio-specific wash fractions were collected.

Succinyl-CoA (0·2 mM) appeared to be the most successful for both enzymes, with respect to percentage recovery and specific activity. In Table 13 (G-STK) a combination of succinyl-CoA + GTP appears to give a higher specific activity but lower percentage recovery, whereas in Table 14 (A-STK) ADP gives a higher specific but lower percentage recovery. Interestingly, in Table 13, it is apparent that adenine nucleotides, although inhibitors of the G-STK enzyme, are able to effect elution of G-STK from the column.

These preliminary screening studies indicate that inclusion of triazine dye chromatography in the purification of G-STK and A-STK from bovine heart should provide a rapid purification step. It is envisaged that, in the future, a mitochondrial preparation followed by dye-ligand chromatography, affinity chromatography and/or FPLC may allow rapid purification of the two enzymes without substantial loss of enzymic activity and degradation.

CHAPTER 4

PROKARYOTIC SUCCINATE THIOKINASES

CHAPTER 4

PROKARYOTIC SUCCINATE THIOKINASES

4·1 Novel STK nucleotide specificity

As previously described in the Introduction, bacterial STKs possess a diversity of nucleotide specificity and molecular size which displays a strong correlation with the taxonomic status of the source organism (Weitzman, 1986). As yet, the biochemical and physiological significance of such diversity is unknown.

Adenine and guanine nucleotides have mainly been used in assaying bacterial STKs, although a few reports have identified that inosine nucleotides can be utilized, along with the former nucleotides, by certain Gram-negative bacterial STKs (Burnham, 1963; Murakami *et al.*, 1972; Kelly & Cha, 1977). Early work on the pig heart G-STK established that it, too, was able to utilize inosine nucleotides (Cha & Parks, 1964). From a report on STK nucleotide specificity in a number of avian tissues (Hamilton & Ottaway, 1981) it was noted that tissues possessing G-STK activity could utilize inosine nucleotides, whereas those tissues with apparently only A-STK activity could not utilize inosine nucleotides. The enzyme phosphoglycerate kinase from baker's yeast has been shown to be able to utilize guanine and inosine nucleotides, whereas the same enzyme from blue-green algae demonstrated absolute specificity for adenine

nucleotides (Söling, 1982). This information, together with the results from the avian tissues (Hamilton & Ottaway, 1981), appeared to indicate a general pattern amongst nucleotide-utilizing enzymes. In the light of the above observations and in connection with the pattern of nucleotide utilization observed amongst bacteria (Weitzman & Jaskowska-Hodges, 1982), it was interesting to re-examine the pattern with inosine nucleotides. Table 15 presents the results of ADP, GDP and IDP utilization in representative organisms of the four main bacterial groups identified by Weitzman & Jaskowska-Hodges (1982). The NDP concentration was set at the Km value of the preferred nucleotide in each case. First, it was seen that IDP and GDP utilization appeared to be closely associated. Secondly, these results were supported by studies on IDP utilization by the separated bovine heart G-STK and A-STK enzymes, purified pig liver G-STK and purified pigeon breast A-STK (Table 16). In every case G-STK enzymes were able to utilize IDP, whereas with A-STK enzymes no activity was observed with IDP. Comparisons of the molecular structures of the nucleotides (Fig.35) suggest that the substituent groups on C-6 of the purine ring may be important in the recognition and binding of the nucleotides to the particular STK enzymes.

However, perhaps the most surprising result displayed in Table 15 was obtained with the Gram-positive bacterium *Bacillus megaterium*. Here, IDP-dependent activity was observed even though no G-STK activity was detectable.

Table 15 IDP utilization by bacterial STKs

Organism	+ ADP	+ GDP	+ IDP
		activity ($\mu mol.ml^{-1}.min^{-1}$)	
A. calcoaceticus (0·05 mM NDP)	0·012	0·135	0·135
E. coli (0·01 mM NDP)	0·128	0·03	0·02
Ps. aeruginosa (0·05 mM NDP)	0·47	0·49	0·48
B. megaterium (0·1 mM NDP)	1·38	0	0·33

NDP concentrations for each organism were set at the Km value for the preferred nucleotide (Weitzman & Jaskowska-Hodges, 1982).

Cells were harvested (13,000 x g for 10 min at 4°C), resuspended in 0·1 M sodium/potassium phosphate buffer, containing 20 % (v/v) glycerol + 1 mM EDTA, pH 8·5, disrupted by Ultrasonication and centrifuged (30,000 x g for 20 min at 4°C)

Table 16 IDP utilization by animal STKs

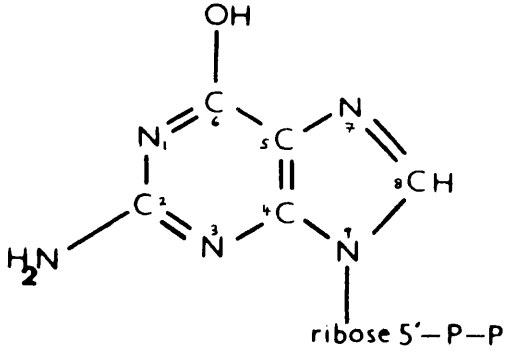
Source	+ ADP	+ GDP	+ IDP
		activity ($\mu mol.ml^{-1}.min^{-1}$)	
Purified pig liver G-STK	zero	0·44	0·35
Purified pigeon breast A-STK	0·28	zero	zero
Separated bovine heart mitochondrial STKs			
G-STK	zero	1·3	1·1
A-STK	0·4	zero	zero

Assay NDP concentrations were 0·5 mM.

Extracts were prepared as in Table 5, page 86.

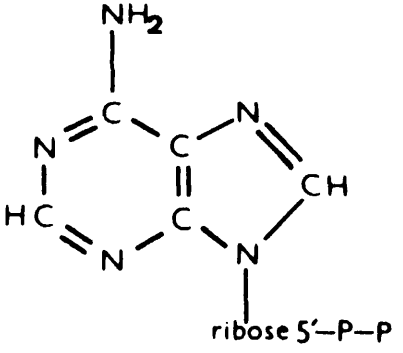
This result indicates either that this particular A-STK, unlike all others, is able to utilize ADP & IDP but not GDP, or that a second STK specific for IDP is present.

Figure 35 Structure of nucleotide diphosphates



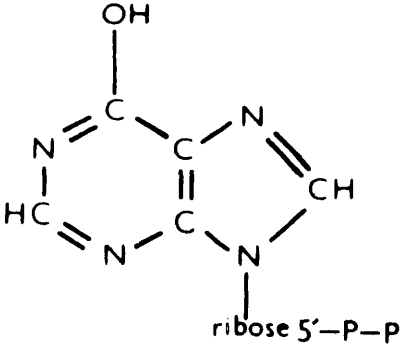
Guanosine 5'-diphosphate

(GDP)



Adenosine 5'-diphosphate

(ADP)



Inosine 5'-diphosphate

(IDP)

4·2 Evidence for two STKs in Gram-positive bacteria

4·2·1 Additivity and differential inhibition of STK activities

The new IDP-dependent STK activity was found to be dependent upon the presence of succinyl-CoA, inorganic phosphate, $MgCl_2$ and IDP in the assay mixture. Table 17 displays results from *B. megaterium* and *B. subtilis*. Partial additivity of A-STK and I-STK activities was clearly seen, similar to the situation with the eukaryotic STKs of Table 5. Significantly, GDP was shown to inhibit I-STK activity (55-64% inhibition), whereas A-STK activity was inhibited to a lesser extent (22-26%). This difference in inhibition by GDP may be due to the structural similarity between GDP and IDP (Fig.35).

4·2·2 pH profile of STK activities

Fig. 36 demonstrates a difference between A-STK and I-STK activities. The pH optimum for A-STK was ~pH 9·2, whereas that for I-STK was ~pH 8·5. This result contrasted with mammalian STKs which both displayed pH optima around pH 8·0. Also, unlike the mammalian STKs, no differential thermal inactivation was observed.

4·2·3 Variation of STK activities on different growth media

The relative specific activities of the two STKs from *B. megaterium* were found to vary depending on the stage of growth and on the carbon source used (Table 18).

Table 17 Additivity and inhibition of Gram-positive STK activities

Assay conditions	Organism	
	B. megaterium	*B. subtilis*
	activity ($nmol.min^{-1}.mg^{-1}$)	
+ ADP	61·3	59
+ GDP	0	0
+ IDP	10·4	12·6
+ (ADP + IDP)	65·3	61·0
% Additivity	91	85
+ (ADP + GDP)	47·8	43·7
% Inhibition	22	26
+ (IDP + GDP)	5·3	6·0
% Inhibition	49	52

0·5 mM NDP used; cells were grown on nutrient broth at 37°C and harvested in early stationary phase.

Extracts were prepared as in Table 15, page 135

Figure 36 pH profile of STK activities from *Bacillus megaterium*.

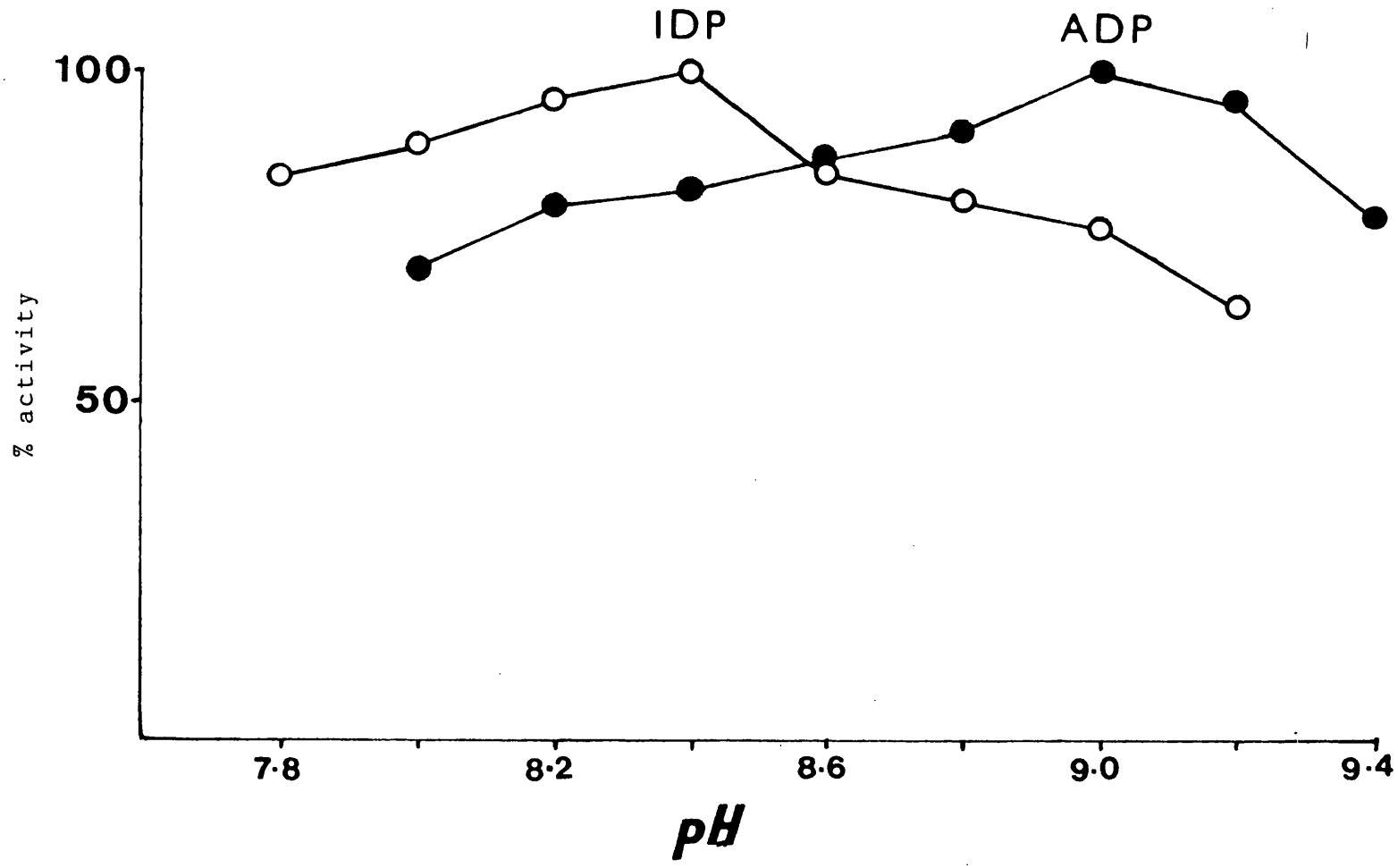


Table 18 Variation in STK activities from *B. megaterium* grown on various media

Growth media	A-STK activity ($nmol.min^{-1}.mg^{-1}$)	I-STK activity ($nmol.min^{-1}.mg^{-1}$)	Ratio A-STK/I-STK
Nutrient broth			
Early - logarithmic	31·0	0·35	88·6
Mid - logarithmic	73·3	4·8	15·3
Late - stationary	48·7	5·6	8·7
25 mM succinate			
Late - stationary	41·1	6·8	6·0
25 mM glucose			
Late - stationary	57·9	1·0	57·9
25 mM glutamate			
Late - stationary	28·1	0·43	65·3

Extracts were prepared as in Table 15, page 135.

On nutrient broth a 10-fold variation in the ratio of A-STK/I-STK occurred between early-logarithmic and late-stationary growth phases, and between different growth media at the same stage of growth. These results, along with those presented in Table 17 and Fig. 36, strongly suggest the presence of two distinct nucleotide-specific STKs, whose relative proportions vary according to physiological conditions. The elevation in I-STK activity during growth on succinate may reflect increased formation of succinyl-CoA from succinate.

B. megaterium, in common with other bacteria, has the ability to store and then utilize poly β-hydroxybutyrate (Dawes & Senior, 1973). The activation of acetoacetate (derived from hydroxybutyrate) by the enzyme 3-oxo-acid CoA transferase requires a supply of succinyl-CoA. *B. megaterium* was therefore grown on acetoacetate and the enzymes of ketone body utilization were measured along with A-STK and I-STK activities (Table 19). Significantly, not only was there a rise in ketone body utilizing enzymes (3-oxo-acid CoA transferase and acetoacetyl-CoA thiolase) but also in I-STK activity. The value of 2·8 for the A-STK/I-STK ratio was the lowest observed from *B. megaterium* grown on any media.

4·2·4 Nucleotide dependence of the STK activities

Figures 37 & 38 display the Hanes-Woolf plots for the STK activities from *B. megaterium* with ADP and IDP respectively. Km and apparent Vmax values ± S·D were

Table 19 Variation in STK, 3-oxo-acid CoA transferase and acetoacetyl-CoA thiolase activities from *B. megaterium* grown on nutrient broth and acetoacetate

Growth media	3-oxo-acid CoA transferase (OAT)	acetoacetyl-CoA thiolase (ACT)	A-STK	I-STK	Ratio A-STK/I-STK
	activity ($nmol.min^{-1}.mg^{-1}$)				
Nutrient broth (Late-stationary)	228	123	44·3	3·8	11·6
25 mM-acetoacetate (Late-stationary)	665	207	39	13·8	2·8

Extracts were prepared as in Table 15, page 135.

Figure 37 Hanes-Woolf plot: [ADP]/v versus [ADP] for A-STK from *B. megaterium* at pH 9·0

Km = 89·4 ± 11·9 µM

Vmax = 0·38 ± 0·02 µmol.ml^{-1}.min^{-1}

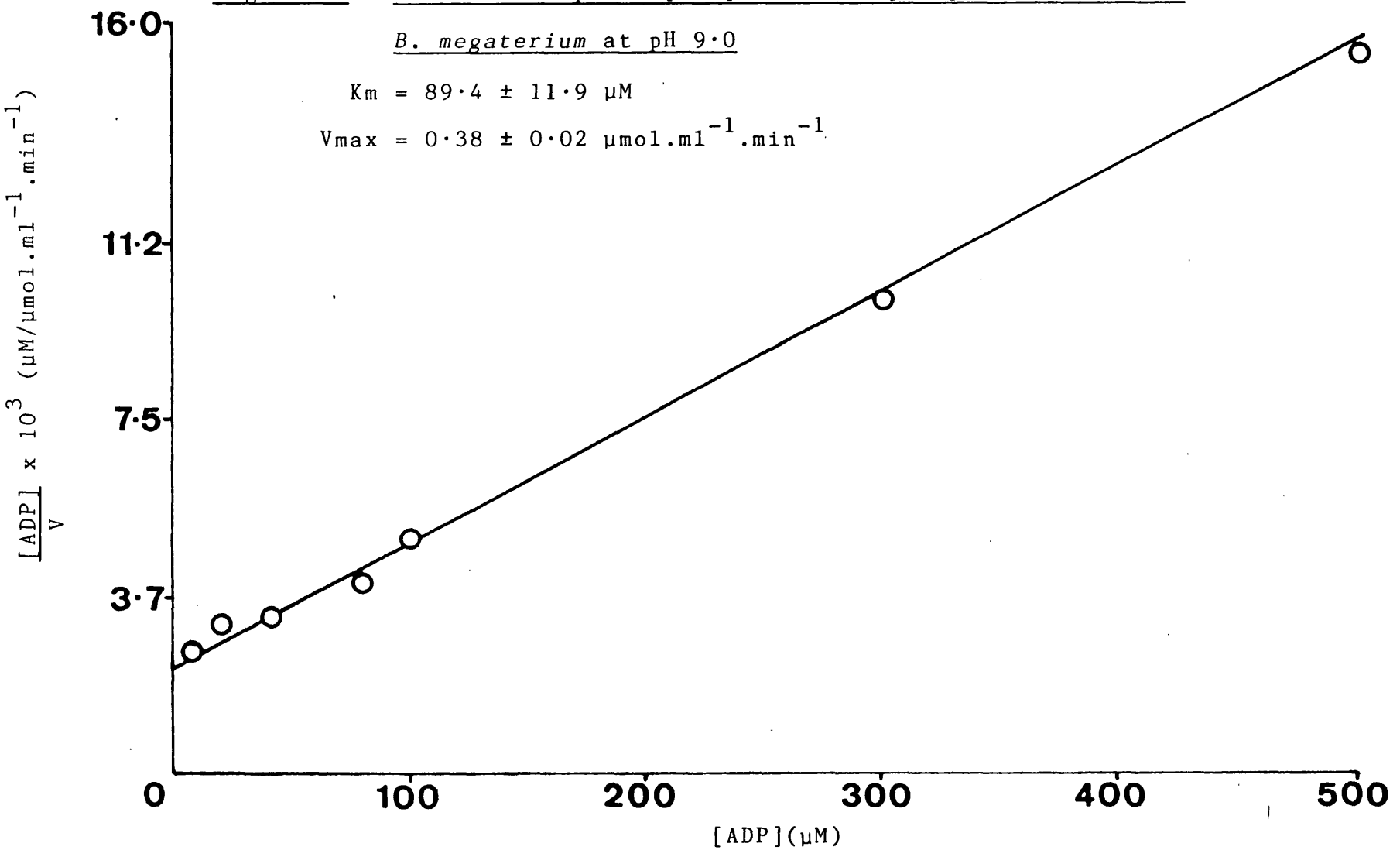
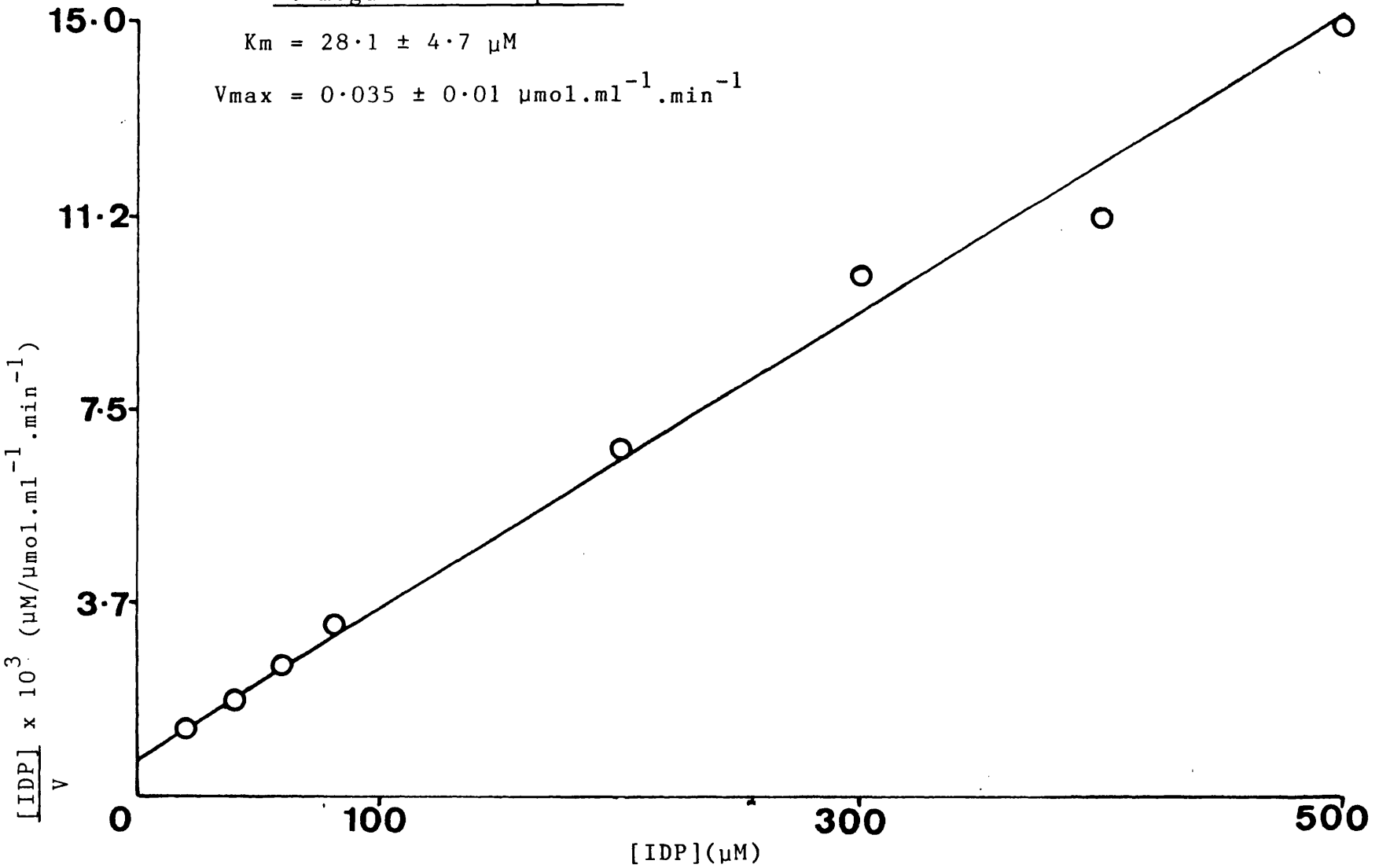


Figure 38 Hanes-Woolf plot: [IDP]/V versus [IDP] for I-STK from *B. megaterium* at pH 8·5



determined using a computer programme of the direct linear plot (Eisenthal & Cornish-Bowdon, 1974).

In contrast to the two distinct STKs found in mammalian tissues, the STKs from *B. megaterium* have different Km values for their respective nucleotides. Km (IDP) = 28·1 ± 4·7 μM, whereas Km (ADP) = 89·4 ± 11·9 μM. This result again indicates the presence of two distinct STKs. Were IDP to be converted into ADP and then react with A-STK, it would be expected that the Km for IDP would be similar to, if not higher than, the Km for ADP. However, the Km for IDP is over 3-fold lower than that for ADP.

4·3 STK activities from a range of Gram-positive bacteria

After observing A-STK and I-STK activities in *B. megaterium* and *B. subtilis*, it was decided to carry out a limited screening of a number of Gram-positive bacteria, to establish the extent of this phenomenon.

It has been suggested that Gram-positive bacteria can be divided into two main groups: group 1, possessing a low G + C DNA e.g. *Bacilli*, *Lactobacilli* & *Brochothrix* and group 2, possessing a high G + C DNA, e.g. *Corynebacterium*, *Kurthia* & *Arthrobacter* (Woese *et al.*, 1985). In Table 20, representatives of the sub-grouping were investigated, with respect to their STK activities: Gram-positive cocci - *Staphylococci* and *Deinococci*; endospore forming rods & cocci - *Bacilli*; non-sporing rods - *Lactobacilli* and *Brochothrix*; coryneform Gram-positive bacteria - *Brevibacterium* and *Corynebacterium* and the related eubacteria - *Kurthia* and *Arthrobacter*.

The original pattern of only A-STK and I-STK activity, as seen in *Bacilli*, was shown to be incomplete. *Brochothrix* and *Deinococci* utilized all three nucleotides, while *Brevibacterium linens* utilized ADP and GDP but not IDP.

Table 20 Nucleotide dependence of STK activities from Gram-positive bacteria

Organism	+ ADP	+ IDP	+ GDP
	activity ($nmol.ml^{-1}.min^{-1}$)		
Low G + C DNA			
Bacillus megaterium	328	36	0
Bacillus subtilis	295	25·8	0
Lactobacillus plantarum	0	0	0
Brochothrix thermosphacta	305	44·8	43·2
Deinococcus radiodurans	79·6	6·0	3·0
Staphylococcus aureus	289	20	0
High G + C DNA			
Arthrobacter simplex	228	16	0
Brevibacterium linens	312	0	156
Corynebacterium fascians	184	147	150
Corynebacterium rubrum	147	80·4	75·5
Kurthia zopfii	36	16·2	0

4·4 Nucleotide dependences and molecular sizes of STKs from Gram-positive bacteria

The Km and apparent Vmax values for ADP, IDP and GDP were determined for the bacteria *Bacillus megaterium*, *Brevibacterium linens* and *Corynebacterium rubrum* (Table 21).

Estimation of the molecular sizes of the STKs from *B. megaterium* and *C. rubrum* were carried out using a calibrated FPLC Superose-12 column, with the addition of LDH (rabbit muscle Mr ~ 140,000) (Figs. 39 & 40). Both Gram-positive bacteria appeared to possess 'small' STK enzymes. In the case of *C. rubrum*, the A-STK gave a Mr value of ~ 87,000, whereas the G/I-STK gave a Mr value of ~ 89,000 (Fig. 39). *B. megaterium* A-STK and I-STK activities gave a similar Mr value of ~ 83,000 (Fig. 40).

These results fall into line with the previously identified pattern of Gram-positive bacteria possessing 'small' STKs (Weitzman, 1981).

Table 21 Km and apparent Vmax values of NDP, for STKs from *Bacillus megaterium*, *Brevibacterium linens* & *Corynebacterium rubrum*.

Kinetic constants	*B. megaterium*		*Brevib. linens*		*C. rubrum*		
	ADP	IDP	ADP	GDP	ADP	IDP	GDP
Km	90	28	28	51	53	43	58
	±10	±4·7	±6·4	±6·5	±10	±8	±15
Vmax (apparent)	0·38	0·085	0·08	0·033	0·14	0·22	0·27
	±0·024	±0·01	±0·001	±0·001	±0·002	±0·013	±0·015

Km values -(μM)

Vmax values -($\mu mol.ml^{-1}.min^{-1}$)

Figure 39 Gel filtration of STKs from *C. rubrum* on FPLC Superose-12 column at room temperature

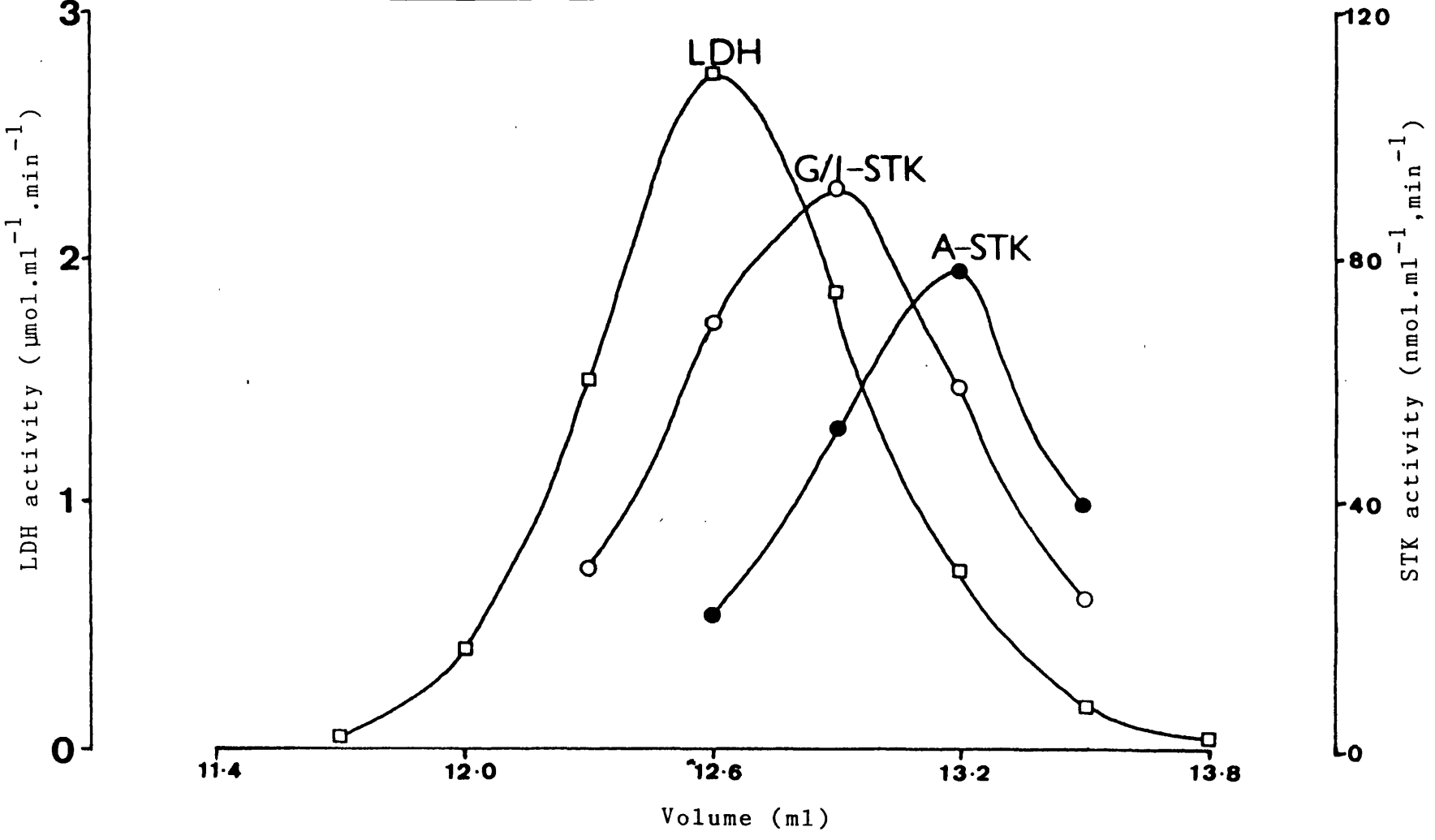
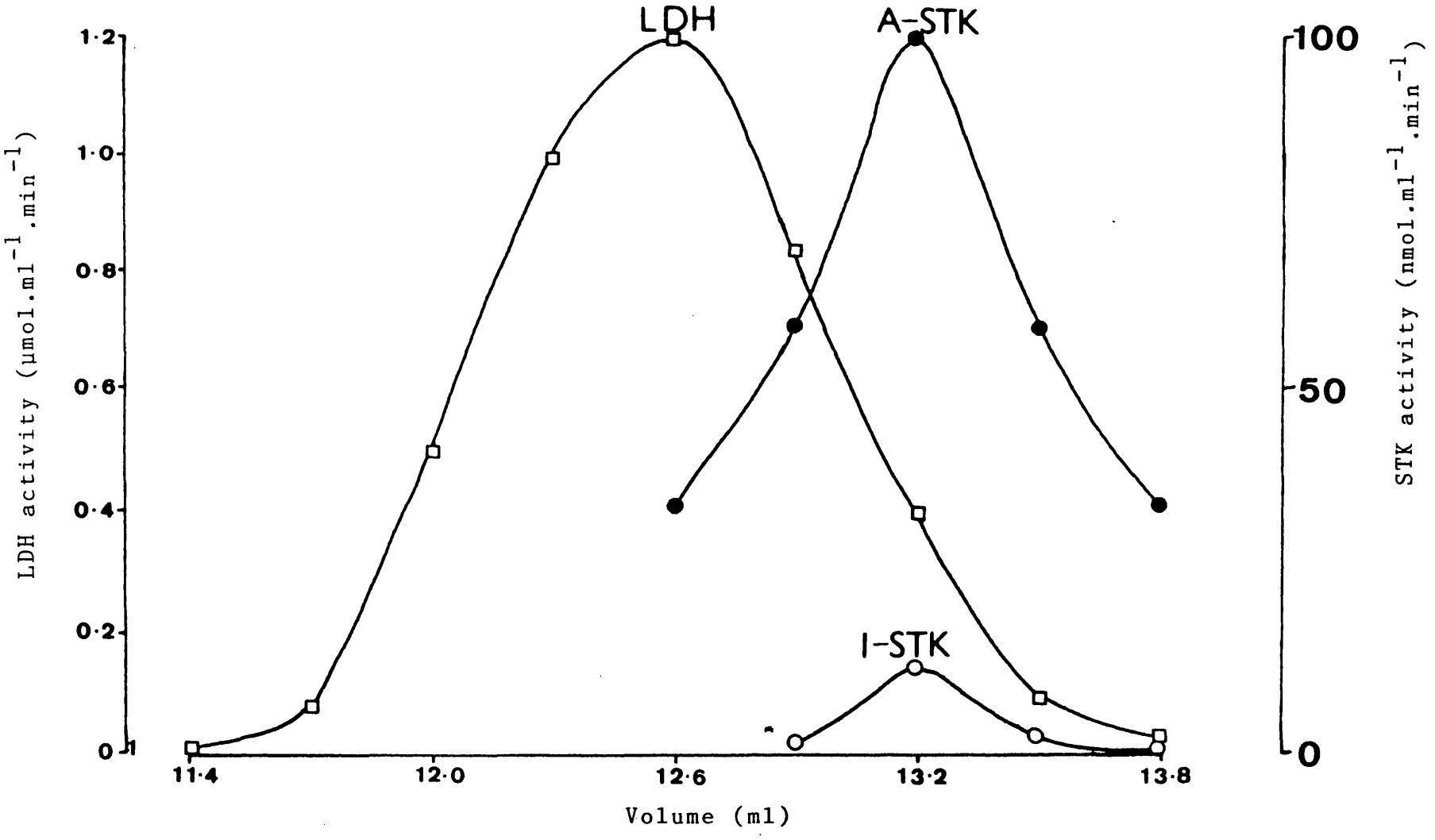


Figure 40 Gel filtration of STKs from *B. megaterium* on FPLC Superose-12 column at room temperature



CHAPTER 5

PHYSIOLOGICAL STUDIES

CHAPTER 5

PHYSIOLOGICAL STUDIES

Following our discovery of two distinct STKs in eukaryotic organisms, one (G-STK) specific for GDP/GTP and the other (A-STK) for ADP/ATP, we addressed the possibility that they serve differing metabolic roles. Succinyl-CoA is known to be involved in three important cellular processes: the citric acid cycle, the activation of ketone bodies and as a precursor for porphyrin biosynthesis. This chapter reports investigations of these three areas with respect to STK activity.

5·1 Ketone body activation

Originally, ketone bodies (acetoacetate and β-hydroxybutyrate) were thought to be the normal intermediates in the degradation of fatty acids. However, after the discovery of Coenzyme A by Lipmann (1945) and subsequent studies (Lynen & Ochoa, 1953) it was established that the CoA-derivatives, not free ketone bodies, were the normal intermediates of fatty acid metabolism. The situation was further complicated when it was shown that L-β-hydroxybutyrate was formed from long-chain fatty acids, whereas the β-hydroxybutyrate that appeared in body fluids was of the D-configuration (Lehninger & Greville, 1953).

Although it was known that circulating ketone bodies

could act as fuel for respiring tissues, they were, in the light of the discoveries in 1953, relegated to an inferior position in metabolism and regarded as waste products. The elevation in plasma ketone bodies, often associated with diabetes, further compounded the view that ketone bodies were undesirable metabolic products.

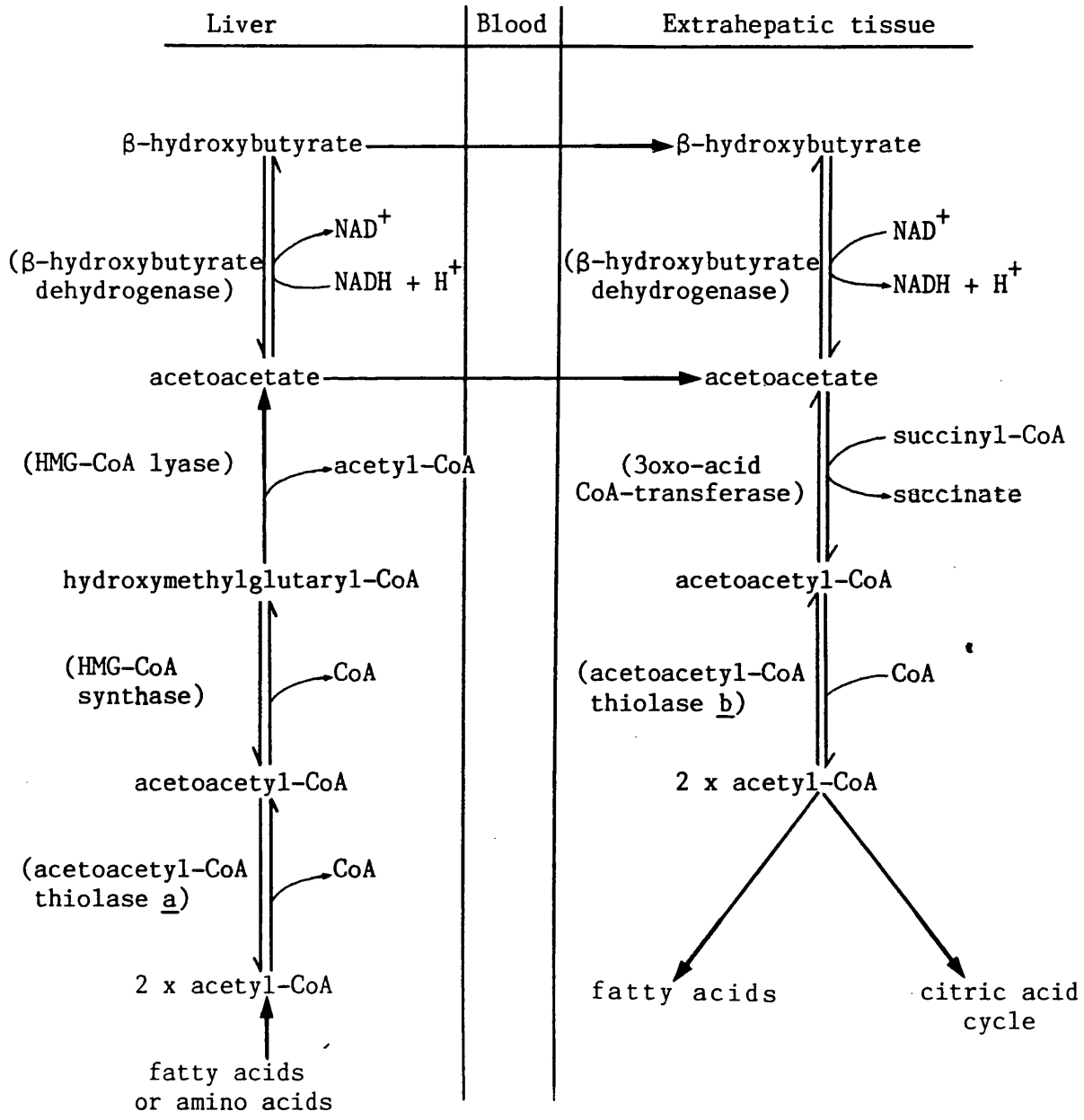
Even twenty years ago, confusion over the reasons for their occurrence was expressed: "Clearly it is not obvious in what ways ketogenesis in fasting is a good thing for the whole animal" (Greville & Tubbs, 1968).

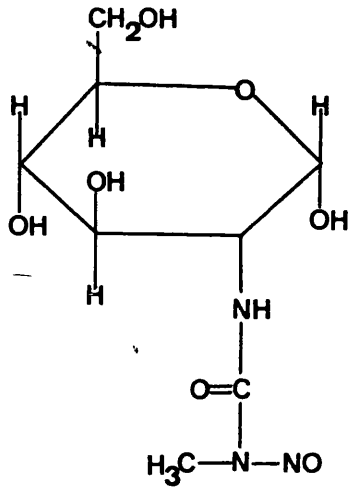
However, in the late 1960s and early 1970s, the significance of the presence of ketone bodies and their physiological roles were elucidated. Today ketone bodies are viewed as having an integral position in the regulation and transport of lipid fuels. They are known to provide alternative fuel to body tissues when carbohydrate is in short supply and this is especially critical for essential muscles such as the heart, diaphragm and digestive tract smooth muscles (Beis *et al.*, 1980). Ketone bodies have even been shown to replace glucose as the major fuel of respiration in the brain, during starvation (Owen *et al.*, 1967) and diabetes (Hawkins *et al.*, 1986). They have also been shown to minimize breakdown of muscle protein, required for gluconeogenesis, during prolonged starvation (McGarry & Foster, 1980) and to act as important precursors for the biosynthesis of neonatal cerebral lipid (Robinson & Williamson, 1980).

Ketone bodies arise from the oxidation of fatty acids and certain amino acids in the liver and can be used by most if not all aerobic tissues (e.g. muscle, brain, kidney, mammary gland and small intestine) with the exception of the liver itself (see Fig. 41). The enzyme hydroxymethylglutaryl-CoA synthase (HMG-CoA synthase), regarded as the rate-limiting enzyme of ketone body production, occurs in large amounts only in the liver. The ketone bodies produced by the liver readily diffuse into the blood and then into extrahepatic tissues. They probably cross the plasma and mitochondrial membranes by diffusion as undissociated acids or enter mitochondria as anions, with a carrier similar to or identical with that used by pyruvate (Robinson & Williamson, 1980). The potential to utilize ketone bodies by extrahepatic tissues (see Fig. 41) is marked by the occurrence of the enzyme 3oxo-acid CoA-transferase (OAT), which is not found in significant levels in liver tissue. This enzyme utilizes succinyl-CoA during the activation of acetoacetate to acetoacetyl-CoA. The occurrence of two STKs in mammalian tissues lead us to study their possible associations with this area of metabolism.

Ketone body metabolism can be perturbed in diabetes and thus offers a means of investigating the participation of the STKs. Diabetes was induced by administration of streptozotocin (Schein *et al.*, 1971).

Figure 41 Ketone body metabolism





Streptozotocin

Streptozotocin, N-Methylnitrosocarbamyl-glucosamine, is an antibiotic derived from *Streptomyces achromogenes*, which has been demonstrated to possess diabetogenic, antitumoral and carcinogenic properties. It is now the compound of choice for producing experimental insulin-dependent diabetes. Streptozotocin induces diabetes by selectively destroying the insulin-secreting β-cells of the Islets of Langerhans. The glucose moiety facilitates entry of the drug into the β-cells, while the N-Methylnitrosourea moiety provides the cytotoxic effect (Fischer, 1985). The mode of action after a single large dose involves alkylation of critical cell components coupled with a more complex damage mechanism. Treatment of pancreatic cells with streptozotocin has been shown to increase the activity of poly (ADP-ribose) synthetase and therefore reduce cellular NAD^+ levels (Okamoto, 1981); such increase in poly (ADP-ribose) synthetase activity is known to be associated with DNA damage. Streptozotocin-

induced DNA damage has been reported in isolated pancreatic cells (Uchigata *et al.*, 1952).

5·1·1 Specific association of STK activity with ketone body utilization

Procedures for the induction and monitoring of experimental diabetes were given in the Methods section 2·10. Both G-STK and A-STK activities were assayed in various tissues from diabetic and control rats (Table 22). The first significant finding is that the G-STK of liver fell in diabetes, but that of brain was markedly elevated. As a consequence, the ratio of G-STK/A-STK in these tissues was greatly changed. Neither heart nor skeletal muscle displayed such marked changes, although a slight depression of both STK activities occurred in the diabetic tissues. As discussed previously, diabetes is accompanied by enhancement of ketone body production by the liver and utilization by the brain (Robinson & Williamson, 1980). The ability of the brain to metabolise ketone bodies had not been believed to be an enzymic adaption, because the enzymes responsible for ketone body utilization - β-hydroxybutyrate dehydrogenase, 3oxo-acid CoA-transferase and acetoacetyl-CoA thiolase - are all present in sufficient activities in the brain and are unchanged by starvation or diabetes (Williamson *et al.*, 1971; Page *et al.*, 1971). It was therefore proposed that only the availability of succinyl-CoA limits the brain's potential to use ketone bodies (Lopes-Cardozo & Klein, 1982). The finding that G-STK increases in diabetic brain suggests that it may produce the succinyl-CoA required for ketone body activation. As mentioned above, diabetes leads to

Table 22 STK activities in rat tissues from diabetic and control animals

Treatment	Tissue	G-STK activity ($nmol.min^{-1}.mg^{-1}$)	A-STK activity ($nmol.min^{-1}.mg^{-1}$)	Ratio G-STK/A-STK
Control	brain	0·33	1·1	0·3
"	heart	4·3	3·0	1·4
"	skeletal muscle	0·6	1·1	0·54
"	liver	4·5	1·5	2·5
Streptozotocin	brain	5·0	2·0	2·5
"	heart	3·7	2·3	1·6
"	skeletal muscle	0·4	1·0	0·4
"	liver	1·3	3·0	0·43

Tissues from 5 control and 5 treated animals were pooled and prepared as in Table 2, page 80.

increased ketogenesis in the liver. The reduction of G-STK in diabetic liver may be related to the recent report (Lowe & Tubbs, 1985) of specific succinylation and inhibition of the enzyme HMG-CoA synthase. This enzyme is crucial in ketone body formation (Fig. 41); its specific inactivation by succinyl-CoA may therefore be a control mechanism in ketogenesis. Stimulation of ketogenesis, e.g. by glucagon, is accompanied by reduction in the concentration of hepatic succinyl-CoA (Seis *et al.*, 1980); this reduction might lead to re-activation of HMG-CoA synthase and hence to increased ketogenesis (Lowe & Tubbs, 1985). The observation of a decreased level in liver G-STK was therefore consistent with a reduction in succinyl-CoA formation and may be related to these other processes. A-STK activity was raised in both diabetic brain and liver (Table 22). In the brain, this may be associated with increased ketone body oxidation via the citric acid cycle, whereas, in the liver, enhanced respiration has been reported to accompany ketogenesis (Seis & Wieland, 1978) and increased hepatic citrate synthase activity is known to occur during starvation and diabetes (Srere, 1968).

Interestingly, it has recently been reported (Boquist & Ericsson, 1986), that streptozotocin has a direct inhibitory action on mouse liver G-STK *in vitro* (50% inhibition at 10 nM streptozotocin), though no *in vivo* alteration in the activity of this enzyme was observed. The effect of streptozotocin was therefore tested on the

activities of G-STK from rat liver and brain. However, no inhibition could be detected even at 100 nM streptozotocin and it may therefore be concluded that the reduction seen in liver G-STK, following streptozotocin administration, was unlikely to be due to any direct inhibition but rather to a reduction in the level of the enzyme.

Table 23 shows the specific activities of a number of enzymes in brain and heart tissues from control and diabetic rats. It is clear that in the diabetic brain there was a general increase in the specific activities, although only G-STK displayed marked elevation. In diabetic heart, 48 h after induction of diabetes, there was a general decrease in the enzyme activities. In the fed state the activities of the ketone body-utilizing enzymes of heart are known to be the highest of all tissues (Beis *et al.*, 1980). However, it has recently been reported (Grinblat *et al.*, 1986) that decreased utilization occurred gradually over the initial period of diabetes (0 - 2 months). This decrease is known to be associated with decreased β-hydroxybutyrate dehydrogenase activity in the inner mitochondrial membrane of diabetic heart.

Ketone bodies are also thought to play an important role in neonatal brain development. At about 8 days after birth, rat neurones are actively extending neuronal processes and forming synapses (a procedure requiring energy and lipid synthesis), while at 15 days most connections have been made and the necessity for utilizing

Table 23 Specific activities of enzymes from control & diabetic rat brain and heart

Enzyme	Control brain	Diabetic brain	Control heart	Diabetic heart
	activity ($nmol.min^{-1}.mg^{-1}$)			
2oxoglutarate dehydrogenase	0	0	23·4	19·2
phosphoenolpyruvate carboxykinase	109	140	76·5	49·2
A-STK	1·0	1·4	3·34	2·9
G-STK	0·27	4·2	4·2	3·8
citrate synthase	10·3	12·7	26·2	21·4
glutamate dehydrogenase	22·9	32·8	12·8	12·9

Tissues were pooled and extracted as in Table 22, page 161.

ketone bodies may have declined (Chechik *et al.*, 1987). Changes in the specific activity of OAT have also been reported (Haney & Patel, 1985), during this period of brain development. This area was investigated with respect to 3oxo-acid CoA-transferase, acetoacetyl-CoA thiolase, citrate synthase and STK enzymes in the developing rat brain. Table 24 presents the specific enzyme activities from rat brain (day 2 - 16). Unfortunately, no convincing trends in OAT or G-STK activities could be identified.

5·1·3 Insulin controlled diabetes

After demonstration of elevated G-STK activity in diabetic brain, further investigations into G-STK association with ketone body metabolism were carried out using insulin administration to diabetic animals and withdrawal of insulin from insulin-controlled animals. Insulin and glucagon (secreted by the α-cells of the pancreas) are mutually antagonistic hormones. Normally, in the controlled state, a fine balance exists between the ratio of the two hormones, which varies depending upon the prevailing circumstances, so that e.g. after a large carbohydrate- or lipid-rich meal, the insulin/glucagon ratio will rise as more insulin is secreted. The main target sites for these hormones are the liver, muscle and adipose tissue. Insulin appears to be responsible for inhibiting ketogenesis, fatty acid oxidation and gluconeogenesis, while activating glycogen formation,

Table 24 Specific activities of enzymes in developing rat brain (day 2 - 16)

Day	3oxo-acid CoA-transferase	acetoacetyl-CoA thiolase	citrate synthase	A-STK	G-STK	Ratio G-STK/A-STK
	activity ($nmol.min^{-1}.mg^{-1}$)					
2	25	13·1	5	0·82	0·44	0·54
7	19·7	10·3	5·7	0·88	1·12	1·3
8	20·3	14	4·2	0·92	0·74	0·8
9	19·4	9	4·5	0·96	0·9	0·94
16	29·8	8·9	9·2	1·3	0·64	0·64

Tissues were pooled and extracted as in Table 22, page 161.

lipogenesis and glycolysis (Agius *et al.*, 1986) whereas glucagon stimulates hepatic gluconeogenesis and ketogenesis, and lipolysis in adipose tissue.

Table 25 presents the body weights and plasma concentrations of β-hydroxybutyrate, acetoacetate and glucose from diabetic animals after administration of insulin. 48 h after induction of diabetes, treated animals presented total ketone body concentrations of 2·55 ± 0·45 mM, whereas the corresponding control values were 0·127 ± 0·04 mM; plasma glucose concentrations were also elevated. Administration of 10 International units of insulin to each rat brought a rapid response in ketone body concentrations in the initial 8 h. However, it took 24 h for glucose concentrations to decrease to control levels. Lipolysis and ketogenesis are known to be much more sensitive to insulin than carbohydrate metabolism (Robinson & Williamson, 1980); therefore ketone body concentrations decrease faster than glucose concentrations. The mild hypoglycaemia seen after 24 h is probably due to administration of excess insulin. These dramatic changes in carbohydrate and lipid metabolism are instigated by discrete enzymic changes. During diabetes the specific activities of the lipogenic enzymes fatty acid synthetase (FAS) and glycerolphosphate acyl-transferase (GPAT) decrease in adipose tissue. Restoration of these activities is observed after insulin administration (Saggerson & Carpenter, 1987). Similar marked enzymic changes occur in carbohydrate metabolism. Normally,

Table 25 Body weight and plasma ketone body and glucose concentrations after insulin administration to diabetic rats

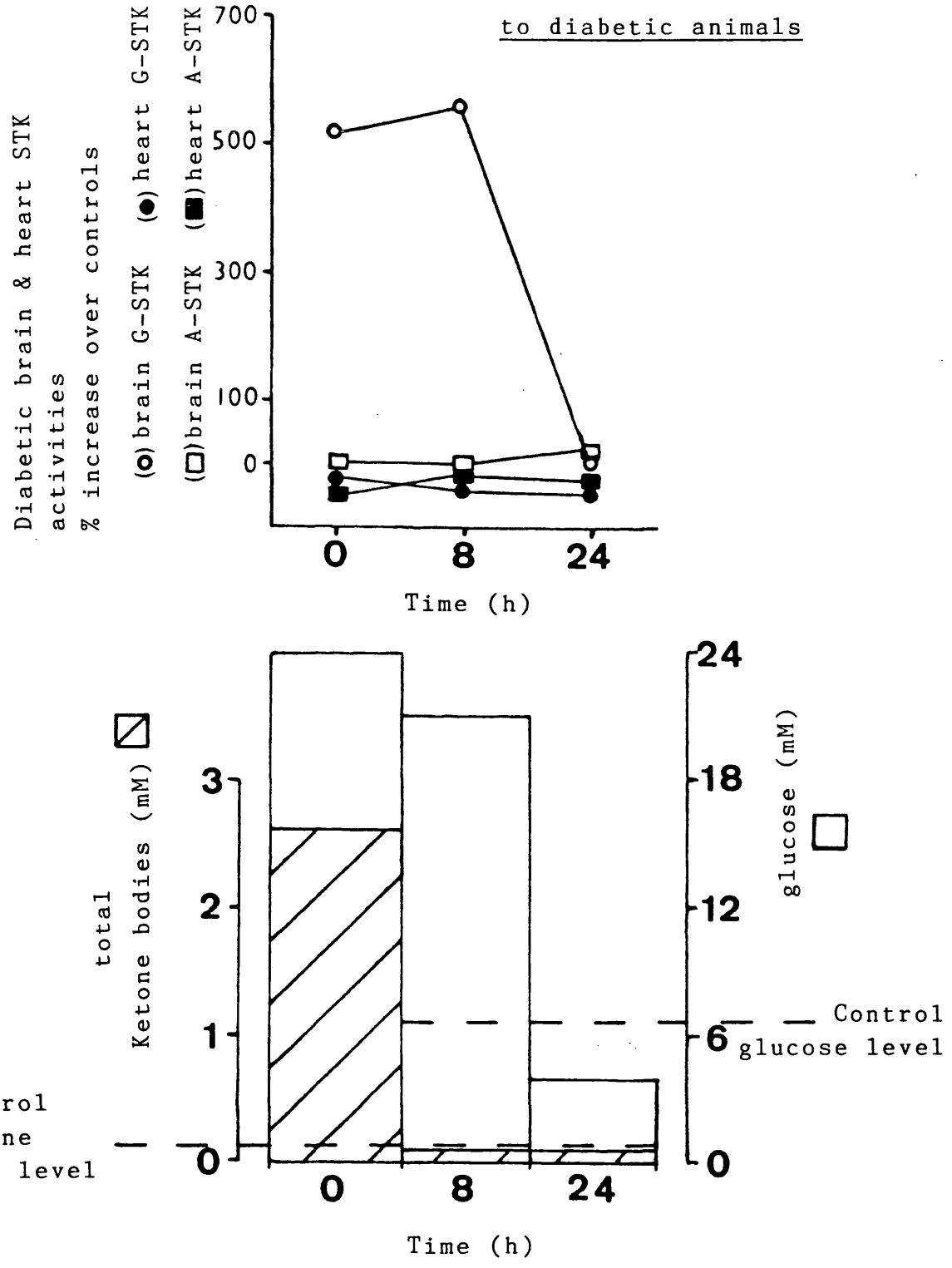
Time after administration of insulin (h)	Body weight (g)		β-hydroxybutyrate (mM)		acetoacetate (mM)		glucose (mM)	
	control	diabetic	control	diabetic	control	diabetic	control	diabetic
0	157·3 ±4·2	133·4 ±9·9	0·057 ±0·01	1·65 ±0·35	0·07 ±0·03	0·09 ±0·1	6·58 ±0·3	24·1 ±1·1
8	161·1 ±6·0	132·0 ±8·0	0·048 ±0·004	0·044 ±0·007	0·054 ±0·007	0·04 ±0·001	7·14 ±0·8	22·8 ±0·3
24	166·0 ±5·5	136·1 ±4·0	0·058 ±0·014	0·049 ±0·02	0·06 ±0·001	0·04 ±0·06	6·8 ±0·56	3·9 ±0·8

skeletal muscle contains mostly hexokinase isoenzyme II, whereas the brain contains predominantly isoenzyme I. However, a marked decrease in isoenzyme II activity occurs in skeletal muscle when insulin is in short supply. This is due to an increased degradation (up to 3-fold) of the enzyme (Frank & Fromm, 1986). Administration of insulin restores hexokinase II activity by reducing the rate of its degradation. During diabetes these changes in the activity of such a key enzyme leads to marked hyperglycaemia because skeletal muscle is quantitatively the most important site for insulin-dependent glucose disposal (Defronzo *et al.*, 1983).

Fig. 42 displays the changes in STK activity in diabetic brain and heart tissues, compared with control values, in connection with the changes in circulating ketone bodies and glucose, after insulin administration. Only diabetic brain G-STK, and not A-STK, shows increased activity concurrent with elevated ketone body utilization. In Fig. 42 this elevation in diabetic brain G-STK was already apparent at the time of insulin administration and persisted for at least 8 h. However, at 24 h, G-STK had returned to the control value. No such changes were observed in diabetic heart tissue, where a slight decrease in both STK activities was observed in the diabetic state (this is similar to results obtained in Tables 22 & 23).

After observing the decrease in STK activities following administration of insulin it was decided to examine the consequences of withdrawal of insulin from

Figure 42 Changes in STK activities, glucose and ketone body concentrations after insulin administration to diabetic animals



Tissues were pooled and extracted as in Table 22, page 161.

insulin-controlled diabetic animals. Fig. 43 displays the changes in body weights associated with induction, insulin -controlled diabetes and then removal of insulin. After receiving streptozotocin on day 1, diabetic animals were then given insulin daily from day 2 onwards. After treated animal body weight values had begun to approach control values, insulin treatment was withdrawn (day 13). The insulin used in the experiment, Neulente-zinc suspension, was active over a 24 h period after administration; therefore no decrease in body weights was observed 24 h after insulin withdrawal. However, after 48 h a marked decrease in body weight was seen. In the subsequent days, body weights continued to decrease but not at such a marked rate. The changes seen in body weights, after insulin withdrawal, were mirrored by changes in ketone body levels (Table 26). A sharp increase in plasma ketone body concentrations occurred concomitant with the marked decrease in body weights (Fig. 43). After 48 h the levels of ketone bodies gradually decreased until, 120 h after insulin withdrawal, their levels were only slightly higher than control values. These changes in ketone body concentrations, at the later part of the experiment, appeared to coincide well with the gradual decrease observed in body weights.

Fig. 44 displays the changes in STK activities that occurred in diabetic brain, after insulin withdrawal, together with the total plasma ketone bodies. 24 h after insulin withdrawal, both G-STK and ketone body concentrations

Figure 43 Body weight changes with and without insulin administration to diabetic animals

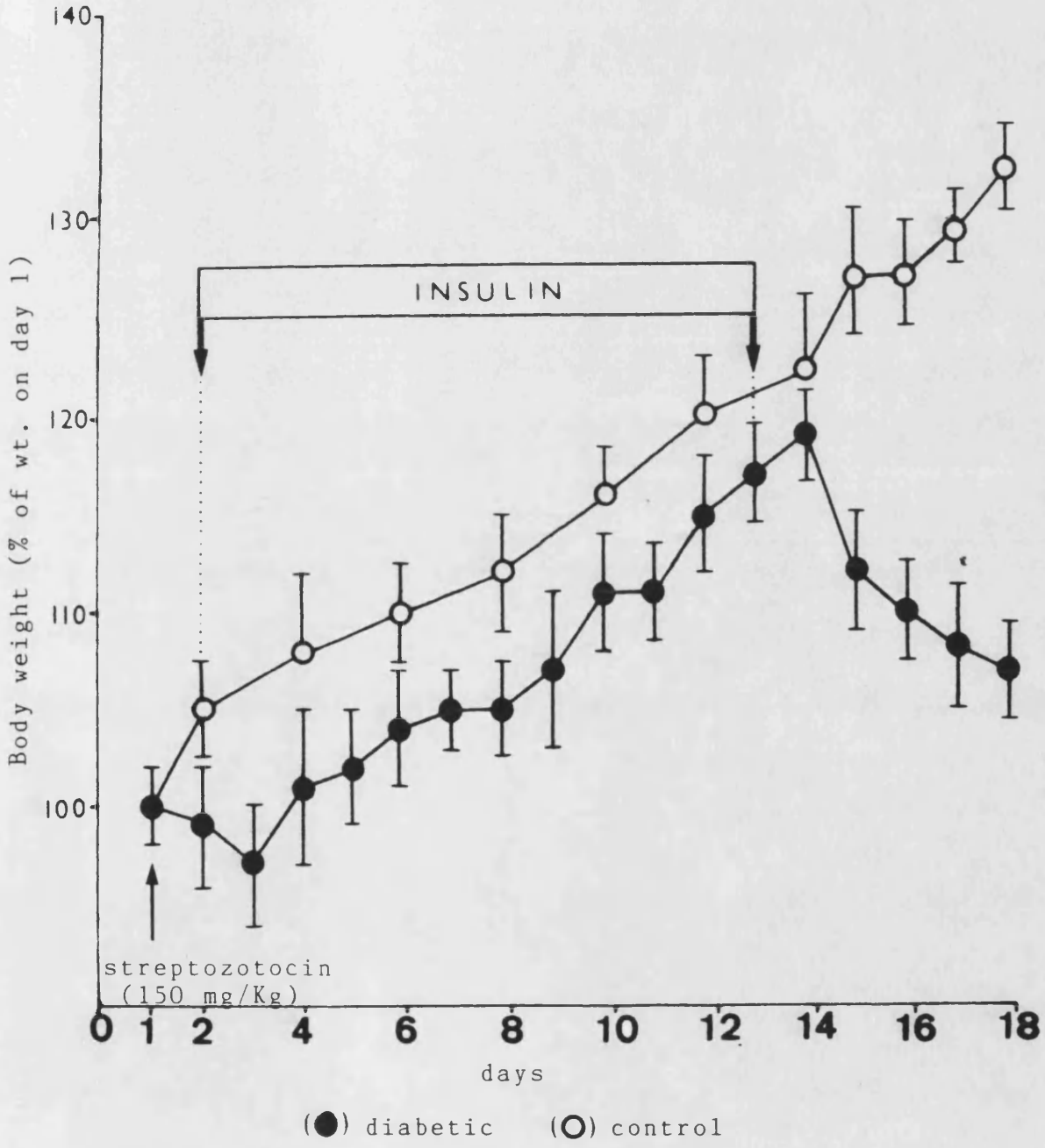
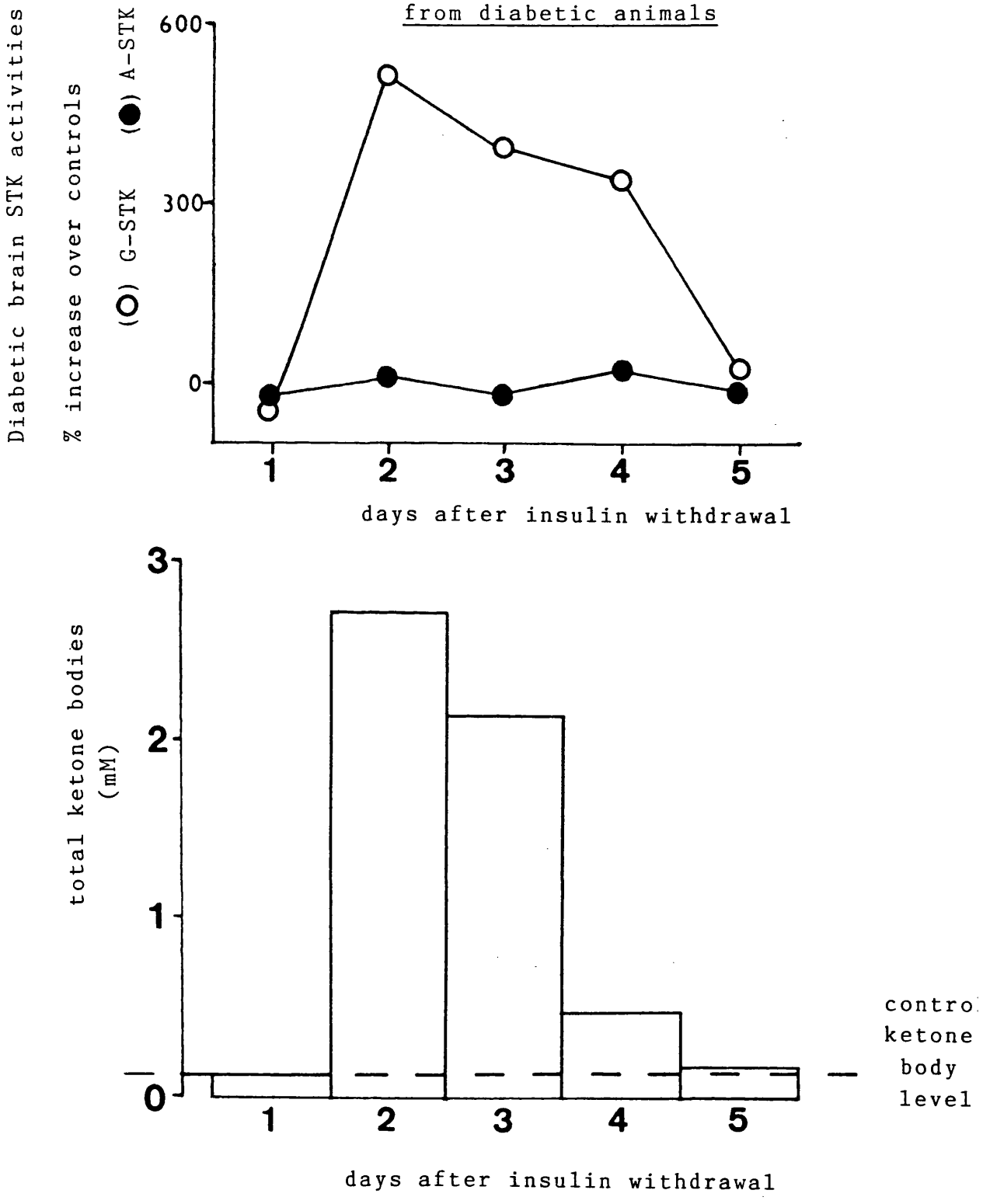


Table 26 Changes in plasma ketone body concentrations after insulin withdrawal from controlled diabetic animals

Time after insulin withdrawal (h)	β-hydroxybutyrate (mM)		acetoacetate (mM)		total ketone bodies (mM)	
	control	diabetic	control	diabetic	control	diabetic
24	0·042 ±0·001	0·057 ±0·007	0·076 ±0·002	0·074 ±0·003	0·12 ±0·003	0·13 ±0·01
48	0·048 ±0·002	1.6 ±0·2	0·068 ±0·004	1·12 ±0·3	0·12 ±0·006	2·7 ±0·5
72	0·047 ±0·001	1·2 ±0·2	0·057 ±0·006	0·85 ±0·06	0·1 ±0·007	2·1 ±0·2
96	0·05 ±0·007	0·33 ±0·03	0·062 ±0·003	0·135 ±0·02	0·11 ±0·01	0·47 ±0·05
120	0·045 ±0·008	0·08 ±0·1	0·059 ±0·005	0·057 ±0·007	0·1 ±0·012	0·14 ±0·02

Figure 44 Changes in STK activities and ketone body concentrations following insulin withdrawal from diabetic animals

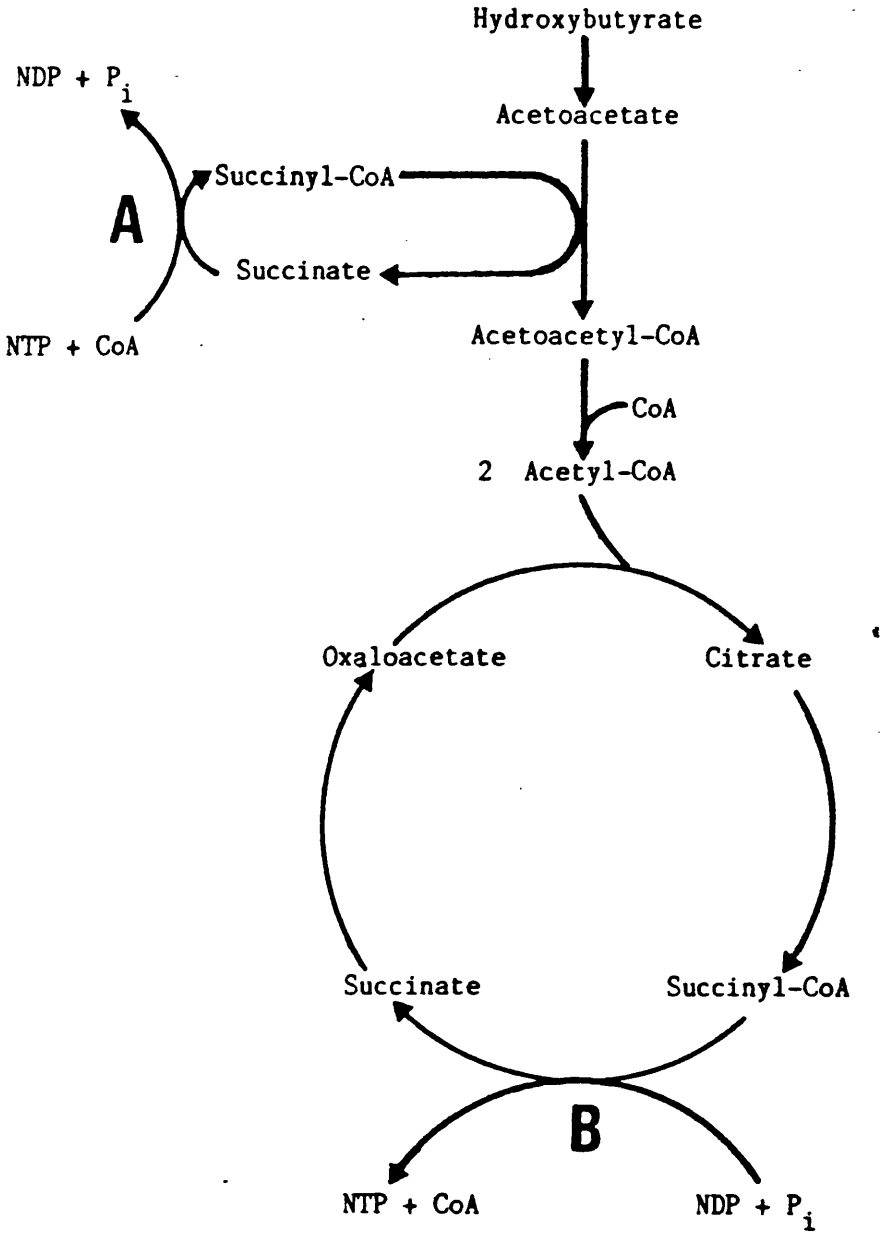


Tissues were pooled and extracted as in Table 22, page 161

were still at control levels. However, elevation of ketone bodies after 48 h was complemented by a similar elevation of G-STK. The levels of G-STK and the available plasma ketone bodies then decreased gradually together until both approached control levels after 5-days.

The results presented in this section support the suggestion that the succinyl-CoA required for ketone body activation is produced directly by the action of G-STK. Fig. 45 shows a scheme of ketone body activation and subsequent oxidative metabolism via the citric acid cycle which is based on the results from this section and from the previously proven presence of a distinct A-STK (see section 3·2). At two points, in the scheme, succinyl-CoA is converted to succinate, by the reaction with acetoacetate (catalysed by OAT) and by reaction with nucleoside diphosphate and inorganic phosphate (catalysed by STK). In the latter case, succinate is further oxidized by the later steps of the citric acid cycle to reform oxaloacetate for condensation with the acetyl-CoA produced from the ketone bodies. In the former case, by contrast, the succinate must be reconverted to succinyl-CoA in order to maintain the flow of acetoacetate to acetoacetyl-CoA. This re-cycling could be achieved either by metabolism of succinate round the citric acid cycle, via oxaloacetate and citrate, or by the action of an STK operating in the reverse direction. It appears from the investigations into STK activities during elevated ketone body utilization that the re-cycling of succinate to succinyl-

Figure 45 Scheme of ketone body utilization



NDP and NTP represent nucleoside diphosphate and triphosphate respectively. A and B are two points at which the succinate thiokinase reaction occurs.

CoA is achieved directly by the action of G-STK. It is therefore possible that the second STK (A-STK) may be associated with the other requirement for the succinate thiokinase reaction, i.e. in the citric acid cycle.

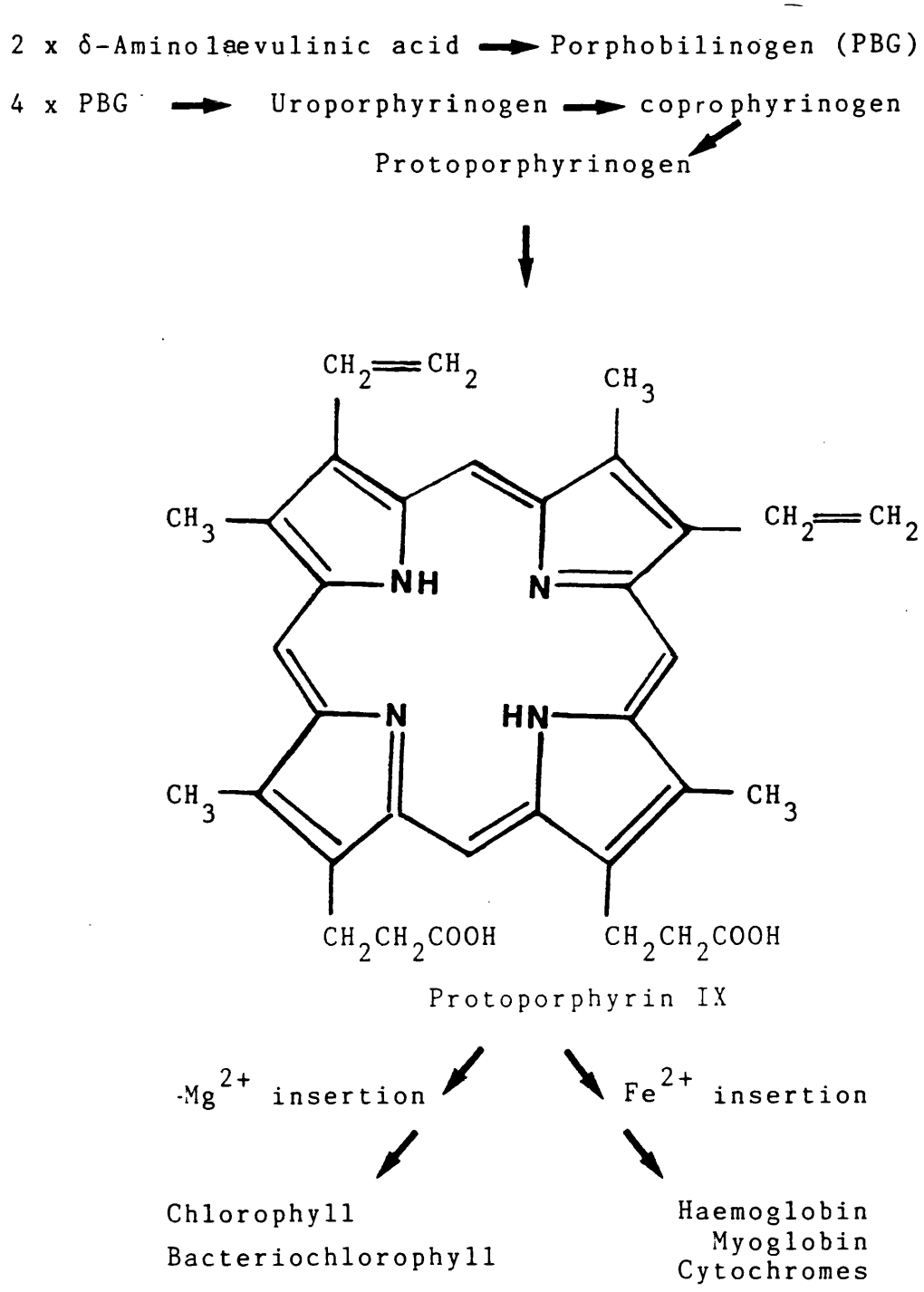
5·2 Porphyrin metabolism

Porphyrins are known to consist of four pyrrole rings (Fig. 46). Early studies, based on structural similarities, suggested proline and the indole ring of tryptophan as possible precursors of the pyrroles (Shemin, 1979). However, following a series of now classical isotope experiments using ^{14}C and ^{15}N, Shemin and co-workers demonstrated that glycine was a precursor of porphyrin (Radin *et al.*, 1950; Wittenburg & Shemin, 1950). Subsequent work revealed that the other carbon atoms of porphyrin were supplied by a succinyl derivative, later identified as succinyl-CoA. Significantly, their work identified that the succinyl-CoA utilized in porphyrin biosynthesis originated directly from succinate, as well as from 2-oxoglutarate dehydrogenase (Shemin & Kumin, 1952). These results therefore demonstrated the existence and reversibility of the succinate thiokinase reaction even before the reaction or enzyme were identified and characterized (Kaufman, 1955; and Sanadi *et al.*, 1956).

Once succinyl-CoA and glycine were shown to be precursors of the pyrrole structure, an intermediate was sought. δ-Aminolaevulinic acid (ALA) was quickly established as the intermediate from which the complete porphyrin structure was synthesised (Shemin *et al.*, 1955). Since that time ALA has been shown to be the first intermediate in the biosynthesis of all naturally occurring porphyrins (Porra & Meisch , 1984).

In animals, ALA is formed by the pyridoxal phosphate-

Figure 46 The biosynthesis of porphyrin



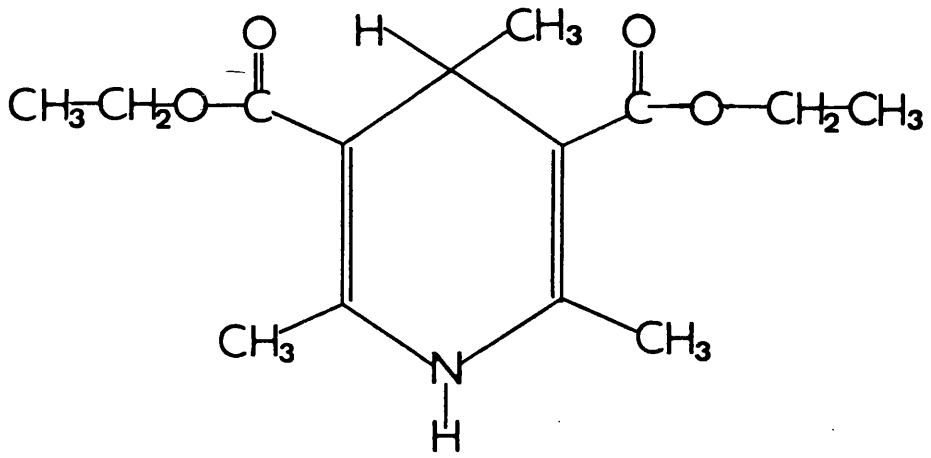
dependent enzyme, δ-aminolaevulinic acid synthase (ALA-S). The synthesis of ALA-S has been found to be under strict negative feed-back control from haem and is regarded as the first and rate-limiting enzyme of haem biosynthesis. In higher plants, algae and cyanobacteria, ALA is not synthesised from glycine and succinyl-CoA, but from glutamate (Castelfranco & Beale, 1983; Oh-homa *et al.*, 1986). The mechanism of this reaction has recently been demonstrated to involve a novel requirement for t-RNA (Schön *et al.*, 1986; Kannangara *et al.*, 1988).

All mammalian cells contain haemoproteins and possess the capability to synthesise haem (May *et al.*, 1986). However, the highest rates of haem biosynthesis are located in the bone marrow of the vertebrae, sternum and pelvis, where erythrogenesis occurs. The liver also actively synthesises haem, which is utilized in the cytochrome P-450 detoxification systems (Ortiz de Montellano & Correia, 1983).

The Porphyrias

A number of disease states of haem biosynthesis occur and are collectively known as the porphyrias (Elder, 1983 b). These perturbations in the normally closely regulated biosynthesis of haem originate from partial deficiency in, or inhibition of, an intermediate enzyme(s) of the pathway. Such conditions have now been identified for each of the steps in the conversion of ALA to haem (Elder, 1983b). These defects do not lead to a deficiency in haem, due to

a compensatory rise in the concentration of the intermediates before the affected enzyme. Each enzyme deficiency produces a characteristic pattern of accumulation and excretion of haem precursors which can cause a variety of clinical symptoms. Porphyrins can be classified into two categories: those in which erythrocyte porphyrin is elevated, and those in which only hepatic porphyrin content is increased. Such increases in the concentrations of the porphyrin intermediates are achieved by elevation of ALA-S activity. Besides genetically inherited porphyria, a wide range of chemicals can induce porphyria (Ortiz de Montellano & Correia, 1983). These drugs and chemicals fall into two main groups. One group, exemplified by the barbiturates, increase haem demand and stimulate the production of microsomal haemoproteins of the cytochrome P-450 series. The second group contains compounds which elevate ALA-S synthesis by inhibiting haem synthesis or by accelerating its degradation. The drug 3,5,-diethoxycarbonyl-1,4-dihydrocollidine (DDC) has been shown to inhibit the final enzyme in the haem biosynthetic pathway, ferrochelatase, which catalyses the insertion of ferrous iron into protoporphyrin IX (Cole & Marks, 1984).



DDC (3,5,-diethoxycarbonyl-1,4-dihydrocollidine)

In the liver of DDC-treated animals, transfer occurs of the 4-methyl group of DDC to one of the pyrrole nitrogens of protoporphyrin IX, converting it to N-methyl-protoporphyrin (De Matteis *et al.*, 1981); this is a potent irreversible inhibitor of ferrochelatase. Probably in the mammalian liver, as in avian liver, decrease in haem production causes derepression of ALA-S synthesis (Elder, 1983 b). This leads to over production of subsequent haem precursors, which can give rise to various clinical manifestations. Such elevation in ALA-S activity must therefore place an increased demand upon succinyl-CoA and glycine within the porphyric liver. Over twenty years ago Labbé *et al.* (1965) demonstrated that induction of porphyria was accompanied by an increase in liver STK

activity.

5·2·1 Specific association of STK activity with porphyrin metabolism

The discovery of two distinct STKs in mammalian tissues prompted a re-investigation of STK activities in porphyric animals. Initially the drug AIA (2-allyl-2-isopropylacetamide) was used in attempts to induce porphyria in rats but this proved unsuccessful. DDC was then used, successfully, to induce porphyria in mice.

Porphyric mouse liver, gall bladder contents and prepared liver mitochondria were all characteristically dark red in appearance. In a typical experiment, total liver porphyrin contents of DDC-treated mice were increased 100-fold from a control value of $\sim$4 nmol g^{-1} liver to a DDC-treated value of $\sim$400 nmol g^{-1} liver, clearly indicating the porphyric condition. Table 27 displays the results from three separate experiments. It is clear that the increase in demand for succinyl-CoA is met by an increase in the activity of G-STK, and not A-STK. Citrate synthase and A-STK activities show no significant changes in activities, whereas G-STK demonstrated a 98% rise in specific activity in the porphyric state compared with control values. In addition to the induction of hepatic porphyria, bovine sternum bone marrow was also investigated, in view of its important role as a site for erythrogenesis. Significantly, sternum bone marrow exhibited the highest G-STK/A-STK ratio of any mammalian

Table 27 Effect of porphyria on citrate synthase and STK activities in mouse liver mitochondria

Treatment	citrate synthase activity ($nmol.min^{-1}mg^{-1}$)	G-STK	A-STK	Ratio G-STK/A-STK
Control liver mitochondria	8·9 ±0·8	3·7 ±0·4	1·5 ±0·5	2·5
DDC-treated liver mitochondria	8·7 ±0·5	7·4 ±1·1	1·6 ±0·3	4·6

Mitochondrial extracts were prepared as in Table 5, page 86. The results presented here represent the mean ($\bar{x}$) of 3 separate experiments ± S.D., with 5 animals per group.

tissue so far examined. The specific activities of G-STK and A-STK were found to be 2·5 & 0·33 $nmol.min^{-1}.mg^{-1}$, respectively, giving a ratio of 7·6. This value is considerably greater than the highest ratio reported for any other tissue e.g. 3·5 from rat kidney (Table 2). This result, taken together with the sharp rise in mouse liver G-STK in the porphyric state, implicates G-STK, and not A-STK, as the STK responsible for supplying succinyl-CoA for haem biosynthesis.

5·3 Life cycle of *Trypanosoma brucei*

Trypanosomes are parasitic protozoa that cause several important human and animal diseases. They are sub-divided into two main groups, the South American Stercoraria (characterized by faecal transmission, e.g. *Trypanosoma cruzi*) and African Salivaria (characterized by salivary transmission, e.g. *Trypanosoma brucei*)(Vickerman, 1985). Trypanosomes possess several unique features, which include microbody-like organelles (glycosomes), which contain the first nine enzymes of glycolysis (Opperdoes & Borst, 1977), a single highly branched mitochondria, and a huge organised network of mitochondrial DNA known as the kinetoplast (Borst & Hoeijmakers, 1979).

The African Trypanosomes, which cause sleeping sickness, have a very remarkable life cycle which includes the ability to evade the host immune system by antigenic variation of surface glycoproteins (Kolata, 1984). *Trypanosoma brucei*, responsible for nagana in cattle, is widely used as the biochemical model for its human pathogenic sub-species (*T. rhodesiense* and *T. gambiense*) (Opperdoes, 1985). During the life cycle of *T. brucei* from mammalian host to insect vector (the tsetse fly), dramatic changes occur in morphology and metabolism (Fairlamb, 1982). In the mammalian bloodstream, *T. brucei* appear to be exclusively dependent upon glucose for energy, being unable to metabolise fatty acids or amino acids due to the absence of a functional citric acid cycle or respiratory chain (Bowman & Flyn, 1976). In the insect

gut, the situation is reversed. Here glucose is scarce and amino acids, such as proline, constitute the main energy source for the procyclic form of *T. brucei* (Broman *et al.*, 1982), proline being oxidized and eventually entering a functional citric acid cycle as oxoglutarate (Ford & Bowman, 1973).

5·3·1 Specific association of STK activity with the citric acid cycle

The bloodstream and procyclic (insect gut) cell forms of *T. brucei* provide an excellent example of a eukaryotic system in which the enzymes of either glycolysis or the citric acid cycle are repressed or derepressed. Both A-STK and G-STK activities had been detected in the bloodstream form of *T. brucei* (see Table 8). We therefore compared the levels of the STKs in both bloodstream and procyclic forms of *T. brucei*, together with key enzymes of the glycolytic and citric acid cycle pathways. The results in Table 28 demonstrate the enzymic changes associated with the differing metabolic requirements of the bloodstream and procyclic cell forms. The specific activities of hexokinase and phosphoglucose isomerase (both glycosomal) and pyruvate kinase (cytosolic) are repressed in the procyclic cell form. Similar changes have also been reported by Hart *et al.* (1984). As predicted, the specific activities of citric acid cycle enzymes increased in the procyclic cell form. Interestingly, citrate synthase activity was detected for the

Table 28 Glycolytic and citric acid cycle enzymes in both bloodstream and procyclic cell forms of *T. brucei*

Enzyme	Bloodstream(B) activities ($nmol.min^{-1}.mg^{-1}$)	Procyclic(P) activities ($nmol.min^{-1}.mg^{-1}$)	Ratio P/B
Citrate synthase	0·34	2·7	7·9
A-STK	5·4	65·0	12·0
G-STK	1·0	1·7	1·7
NADP-Isocitrate dehydrogenase	3·4	28	8·2
Malate dehydrogenase	130	1800	13·8
Hexokinase	460	13·5	0·03
Phosphoglucose isomerase	500	74	0·15
Pyruvate kinase	30·5	9·4	0·31

Pellets of both cell forms were resuspended in 0·1 M sodium/potassium phosphate buffer, containing 20 % (v/v) glycerol, pH 7·5, disrupted by Ultrasonication and centrifuged (5000 x g for 2 min at room temperature).

first time in the long slender bloodstream form of *T. brucei*. Succinate dehydrogenase now appears to be the only enzyme of the citric acid cycle absent in the bloodstream form, with the remaining enzymes of the cycle all attenuated (Ryley, 1962; Overall *et al.*, 1986). The results in Table 28 indicate that the specific activities of the citric acid cycle enzymes increase in concert from the bloodstream to procyclic forms. Significantly, it is A-STK and not G-STK which displays this marked elevation, thus clearly implicating A-STK as a component of the cycle.

5·3·2 Occurrence of ketone body-utilizing enzymes in both forms of *T. brucei*

After identification of the associations of G-STK with ketone body utilization and porphyrin biosynthesis, the occurence of G-STK activity in both forms of *T. brucei* required explanation. Trypanosomes are thought not to be able to synthesise haem, so equine haemin has always been included in their growth media. Yet this area of their metabolism has not been thoroughly investigated and it has been suggested that the inclusion of haemin may not be essential (W. Gibson, personal communication). In the case of ketone body utilization no investigations have been reported. The presence of ketone body-utilizing enzymes was therefore sought in both forms of *T. brucei*. Table 29 shows the existence of 3-oxoacid CoA-transferase (OAT) and acetoacetyl-CoA thiolase (ACT) activities in both forms of *T. brucei*. This is the first time that the

Table 29 Ketone body-utilizing enzymes in *Trypanosoma brucei*

Cell form	3 oxo-acid CoA-transferase activity ($nmol.min^{-1}.mg^{-1}$)	acetoacetyl-CoA thiolase
Bloodstream	1·22	1·1
Procyclic	3·2	2·3

Bloodstream - total cell $N\bar{o}$ = 4·3 x 10^9 cells

Procyclic - total cell $N\bar{o}$ = 3 x 10^9 cells

Extracts were prepared as in Table 28, page 188

enzymes required for utilizing ketone bodies have been reported in *T. brucei*. These results are relevant to the recent identification of citrate synthase activity in the bloodstream form of *T. brucei*. The bloodstream form is known not to contain pyruvate dehydrogenase activity. It is therefore conceivable that the acetyl-CoA utilized by the newly identified citrate synthase may originate from the metabolism of ketone bodies.

Further attempts were made to clarify the association of G-STK with ketone body utilization in *T. brucei*. Table 30 presents the specific activities of a number of citric acid cycle enzymes, together with the activities of the STKs and ketone body-utilizing enzymes, from procyclic cells grown in normal medium and normal medium + 10 mM acetoacetate or β-hydroxybutyrate. Although the results are not as convincing, concerning association of G-STK with ketone body utilization, as in section 5·1, the only enzymes that show a significant elevation are G-STK, 3-oxoacid CoA-transferase and acetoacetyl-CoA thiolase. The possible reasons for the presence of ketone body-utilizing enzymes in both forms of *T. brucei* will be discussed in section 6·4.

Table 30 Citric acid cycle and ketone body-utilizing enzymes from procyclic cells grown in normal medium and medium supplemented with acetoacetate or β-hydroxybutyrate

Enzyme	Normal medium	+ 10 mM acetoacetate	+ 10 mM β-hydroxybutyrate
	activity ($nmol.min^{-1}.mg^{-1}$)		
Citrate synthase	3·8	1·9	2·1
NADP-isocitrate dehydrogenase	27	22	26
2-oxoglutarate dehydrogenase	11·2	12·1	8·2
malate dehydrogenase	1180	1000	1000
A-STK	49	35·4	40·1
G-STK	1·3	2·3	2·3
3-oxoacid CoA-transferase	1·1	1·5	1·6
acetoacetyl-CoA thiolase	1·3	2·4	2·0

Total cell counts - Normal medium = $2·55 \times 10^9$ cells
+ acetoacetate = $1·15 \times 10^{10}$ cells
+ β-hydroxybutyrate = $1·01 \times 10^{10}$ cells

NB The ketone body supplements were added to established cultures 24 h before harvesting.

Extracts were prepared as in Table 28, page 188

CHAPTER 6

GENERAL DISCUSSION

CHAPTER 6

GENERAL DISCUSSION

6·1 Introductory comments

As was emphasised in the Introduction, the vast majority of studies on STK have been concerned with the mechanistic and molecular aspects of the enzymes from *E. coli* and pig heart. The detailed molecular attributes of both these representative STKs continues to be elucidated (Wolodko & Bridger, 1987; Nishimura *et al.*, 1988; Khan & Nishimura, 1988). However, very little attention has been focussed upon the possible metabolic significance of the diversity of molecular size and nucleotide specificity displayed by STK throughout Nature (Weitzman, 1981). Only one publication occurs in the literature, a minireview entitled "STK and metabolic control" (Ottaway *et al.*, 1981), which attempts to assess and rationalise the STK nucleotide specificity displayed by a tissue/organism in relation to the metabolic constraints placed upon that tissue/organism. The extent to which the observations and predictions of that paper correlate with the findings presented in this thesis will be discussed fully in sections 6·4 & 6·5.

Finally, in this opening section, it is perhaps appropriate to comment upon the possible reasons why the discoveries reported here were not observed by earlier workers. First, the initial confusion concerning the

nucleotide specificity of pig heart G-STK (Kaufman, 1951; Sanadai *et al.*, 1956) and the subsequent identification of G-STK activity in a number of mammalian tissues, may have influenced investigators to assay only for G-STK and to interpret any STK activity observed with adenine nucleotides as arising from the presence of nucleotide diphosphokinase (NDPK) activity. Secondly, our findings were greatly facilitated by our use of the polarographic method of enzyme assay (Weitzman, 1976). As discussed in Methods section 2·4·5, the use of the polariter was particularly suitable for examining the nucleotide specificity and dependency of STKs. The majority of investigators employ the discontinuous hydroxamate method (Kaufman, 1955) for determination of STK activity, even though as early as 1964, hydroxylamine was demonstrated to be a competitive inhibitor of STK with respect to CoA-SH (Cha *et al.*, 1964). Finally, the ability to measure polarographically the activity of STKs in the most turbid of extracts permits the detection of STK activities even when the various spectrophotometric methods are unsuitable.

6·2 Eukaryotic STKs

6·2·1 Distinct STKs

The discovery of the existence of two distinct STK enzymes, within animal tissues, which possess strict specificity for their respective nucleotides is one of the most significant findings presented in this thesis. The subsequent studies concerning prokaryotic STKs and the metabolic roles of eukaryotic STKs were prompted by the discovery of two distinct enzymes.

The occurrence of A-STK and G/I-STK enzymes appears to be a general phenomenon throughout the eukaryotic organisms examined. In contrast to Ottaway and co-workers (Ottaway *et al.*, 1981), both enzyme activities were found in all mammalian tissues, locust flight muscle and pigeon breast muscle. Both activities were also detected in toluenized yeast cells, zea maize mitochondrial and *Trypanosoma brucei* cell extracts.

The possibility that the detection of STK activities linked to adenine nucleotides (ATP/ADP) may be artifactual, due to the presence of NDPK activity, was addressed. However, this hypothesis was rejected, first because in a number of tissues (i.e. brain & skeletal muscle) A-STK activities were greater than G-STK activities and, secondly, because of the ability to obtain additivity of both the enzyme activities in a single source. Hamilton & Ottaway (1981) reported the presence of GTP-dependent and ATP-dependent STK activities, yet remained uncertain as to whether they were dealing with a system containing two

enzymes or an enzyme of broad specificity; however they emphasised that the presence or absence of NDPK had no effect on the ratios of the G-STK/A-STK activities they observed. The inclusion of EGTA in mitochondrial extraction buffers, known to effect removal of NDPK activity from mitochondria (Pedersen, 1973), effectively rules out any NDPK involvement. Even *in vivo*, the significance of NDPK on the STK system and equilibrium between intramitochondrial nucleotides has been questioned (Ottaway *et al.*, 1981). In our studies no changes in the G-STK/A-STK ratios could be seen even between crude tissue and washed mitochondrial extracts. The fact that very little, if any, NDPK activity occurs within the mitochondrial matrix (Pedersen, 1973) makes it difficult to understand biochemical text-book assertions that the GTP produced by the substrate-level phosphorylation step of the citric acid cycle is used to rephosphorylate ADP, by the action of NDPK (Stryer, 1988).

The demonstration and then separation of G-STK and A-STK enzymes from animal tissues might of course reflect cell heterogeneity within the tissues; such a claim has been made for hepatic parenchymal cells (Jungermann & Sasse, 1978). That this is unlikely to be the explanation is supported by our examination of two cell lines - mouse lymphoma L1210 and baby hamster kidney (BHK) cells. Extracts of both these cell types displayed GDP-dependent and ADP-dependent STK activities, indicating the presence of both enzymes within single cells. Moreover, the ratio

of G-STK/A-STK in the BHK cell line was similar to that obtained in kidney tissue extracts.

Throughout this investigation no evidence was obtained for the occurrence of any extra-mitochondrial STK activity in eukaryotic organisms, apart from the traces of A-STK and citrate synthase activity detected in zea maize etioplasts, a finding that will be commented upon in section 6·4.

The distinct nucleotide specific STKs found in all animal tissues appear to be very similar, apart from tissue distribution and stability variations, with respect to molecular size, pH optimum and substrate affinities. All eukaryotic STKs examined by previous investigators were 'small' ($Mr < 100,000$) (Weitzman, 1987). In the present work, the A-STK and G-STK enzymes from bovine heart, pigeon breast muscle and pig liver were all shown to possess molecular weights estimated at between 79,000-85,000.

Upon separation of A-STK and G-STK activities from bovine heart mitochondria, it was noticed that neither of the separated enzymes displayed any activity whatsoever with the other nucleotide. In fact the activities of both A-STK and G-STK were shown to be inhibited by the presence of GDP and ADP respectively. This result explained why only partial additivity of both STKs was observed when activity measurements were made in extracts in the presence of both nucleotides. Interestingly, ADP was shown to be a competitive inhibitor, with respect to

GDP, for the G-STK, possessing a K_i value of $\sim$230 μM. As the concentration of ADP within the mitochondrial matrix is probably of the order of 1-10 mM (Soboll *et al.*, 1978), compartmentation of the STKs and their respective nucleotide substrates must be occurring to allow operation of G-STK.

6·2·2 Purified STKs

The purifications of pig liver G-STK and pigeon breast A-STK, both relatively rich sources of each enzyme, were carried out with a view to devising procedures for eventually purifying both enzymes from a single source, e.g. bovine heart mitochondria. An adenine nucleotide-specific STK had not previously been purified to homogenicity from any eukaryotic source, despite a recent attempt to purify A-STK from baker's yeast (Schwartz *et al.*, 1983). Soon after the establishment of a purification procedure for pigeon breast A-STK, Allen & Ottaway (1986) published a procedure for partial purification of A-STK from pigeon breast muscle. Significantly, their 80% purified A-STK (in terms of protein) had a specific activity of 1·5 units mg^{-1} protein, which is near the value of 1·83 units mg^{-1} protein that was obtained for the electrophoretically homogeneous enzyme. These similar results confirm the relative low activity of pigeon breast A-STK in comparison with the high oxidative capacity of pigeon breast muscle (Allan & Ottaway, 1986). The purification of pigeon breast A-STK required

hydroxylapatite, affinity and fast protein liquid chromatographic procedures, whereas the purification of pig liver G-STK did not require the final FPLC procedure. The specific activity of the pig liver G-STK was close to that obtained for the rat liver G-STK (Ball & Nishimura, 1980), although higher specific activities have been reported for the pig heart G-STK (Brownie & Bridger, 1972). In general, the overall purification yields of homogeneous STK enzymes from eukaryotes have been low (Nishimura, 1986), and this was also reflected in the yields obtained for the purified enzymes from pig liver and pigeon breast.

As previously discussed (section 3·7), initial attempts to purify A-STK and G-STK from a single source were unsuccessful. However, the preliminary results from the screening of the triazine-dye columns and subsequent bio-specific elutions, provides a foundation for future purification, which will then allow direct molecular comparisons.

With the establishment of clear evidence for two distinct STKs in animal tissues, we then addressed the intriguing possibility that they may serve differing metabolic roles. The results of physiological studies are discussed in section 6·4.

6·3 Prokaryotic STKs

Novel nucleotide specificity

Gram-negative bacteria, in contrast to Gram-positive bacteria, possess a 'large' citrate synthase (a hexamer of identical subunits) and a 'large' STK (an $\alpha_2\beta_2$ tetramer) (Weitzman, 1981). During our studies, on a number of Gram-negative bacteria, no evidence was found for the existence of more than one STK enzyme; rather, under whatever growth conditions or media used (e.g. *Rhodopseudomonas spheroides* grown aerobically or anaerobically), no changes in the ratio of GDP-dependent or ADP-dependent STK activities were detected. These results were in line with the pattern that 'large' STKs are able to utilize guanine and adenine nucleotides, whereas the 'small' STKs found in eukaryotes and Gram-positive bacteria are specific for either adenine or guanine nucleotides.

Previously, only A-STK activities have been found in Gram-positive bacteria (Weitzman & Jaskowska-Hodges, 1982). Our discovery of an STK of novel nucleotide specificity was therefore most surprising. As previously discussed (section 4·1), guanine and inosine nucleotide utilization was considered to be characteristic of all G-STKs, with only A-STKs being strictly specific for adenine nucleotides. However, in a number of Gram-positive bacteria, an STK activity strictly specific for IDP occurred, which was in fact inhibited by GDP. These observations were significant because, prior to this

discovery, Gram-positive bacteria were the only organisms apparently unable to display STK activity with at least two nucleotides. It therefore now appears that all organisms possess the capability to utilize adenine, guanine or inosine nucleotides during STK activity.

6·3·2 Distinct STKs

After the detection of IDP-dependent and ADP-dependent STK activities from *Bacillus megaterium*, attempts were made to separate the two STK activities. Unfortunately, all such attempts (i.e. using affinity chromatography and FPLC procedures) proved unsuccessful due to the instability of enzyme activity. The inclusion of glycerol, protease inhibitors and reducing agents all failed to stabilize the activities. However, indication of the existence of distinct STK enzymes was obtained from additivity and optimum activity pH profile studies. These results were complemented by comparative enzyme activity and nucleotide dependence studies. A 10-fold change in the A-STK/I-STK ratios was obtained with *B. megaterium*, between early logarithmic and early stationary phases during growth on nutrient broth. This change originated from a rise in I-STK specific activity rather than a marked change in A-STK specific activity. Such wide changes in the ratio of A-STK/I-STK activities were also observed when *B. megaterium* was grown on other carbon sources, such as succinate and glucose. The arguments rehearsed when proposing distinct STKs in animal tissues

were also valid in this system. The occurrence of two distinct STKs, each specific for its own nucleotides, was a simpler explanation than that of a single enzyme whose nucleotide specificity changed, depending upon the growth phase of the bacterium or the carbon source being utilized.

Perhaps the strongest indication of a distinct I-STK enzyme was obtained when the K_m values for IDP and ADP were compared. The lower K_m value, obtained for IDP, ruled out any possibility that IDP was converted to ADP, thus providing a substrate for A-STK.

The occurrence of differences in STK ratios is indicative of the STKs playing differing metabolic roles within the bacterium. Identification of an STK specifically utilizing inosine nucleotides poses questions as to the provision of IDP/ITP and the possible participation of inosine nucleotides in metabolism. Very little is known about inosine nucleotides in general metabolism, apart from the involvement of IMP in the biosynthesis of adenine and guanine nucleotides and the occurrence of inosine in the anticodon triplet of yeast alanine t-RNA (Lehninger, 1975). Thus, although inosine nucleotides are known to be able to replace guanine nucleotides in many enzyme reactions, they are still regarded as the 'poor relations' among nucleotides. It may well be that, just as guanine nucleotides play a more significant role in cellular metabolism than was once thought, inosine nucleotides may also be involved in a range of other processes.

The screening of a variety of Gram-positive bacteria, with respect to their STK nucleotide specificities, revealed that a number of bacteria displayed similar A-STK and I-STK activities to those found originally in *B. megaterium*. However, certain bacteria displayed STK activities with only ADP and GDP, or with all three nucleotides (ADP, GDP & IDP), e.g. *Corynebacterium rubrum*. Interestingly, *C. rubrum* appears, on the basis of molecular weight, to possess distinct A-STK and G/I-STK enzymes. It is therefore apparent that, despite earlier assertions, Gram-positive bacteria do indeed possess more than one STK enzyme.

6·4 Physiological studies

"It is not unusual to find that where a chemical reaction serves more than one purpose in the cell, the enzyme catalysing that reaction is synthesised in multiple forms" (Kornberg, 1970).

The discovery of two distinct enzymes catalysing the same basic reaction poses the possibility that the enzymes may be responsible for operating the reaction in opposite directions. Within many organisms, succinyl-CoA is involved in three main metabolic processes: first in the citric acid cycle, secondly in ketone body activation and finally as a precursor of porphyrin. Even though the succinate thiokinase reaction is regarded as an equilibrium reaction, the operation of the citric acid cycle would cause the reaction to operate in the direction of succinyl-CoA hydrolysis, whereas during ketone body activation or porphyrin biosynthesis, if the succinate thiokinase reaction were involved, it would operate in the direction of succinyl-CoA formation.

In the case of porphyrin biosynthesis, evidence for the formation of the required succinyl-CoA direct from succinate was obtained early in the elucidation of the porphyrin biosynthetic pathway (Shemin & Kumin, 1952). However, much confusion still exists concerning the role of STK, in relation to the activation of acetoacetate and operation of the citric acid cycle.

6·4·1 Ketone body activation

Ottaway and co-workers (McClellan & Ottaway, 1980; Hamilton & Ottaway, 1981; Ottaway *et al.*, 1981) have suggested that ketone body utilization requires the partitioning of succinyl-CoA (formed by 2-oxoglutarate dehydrogenase) between 3-oxoacid CoA-transferase (OAT) and STK. The K_m for succinyl-CoA is very much lower for STK (~30-50 μM) than for OAT (4·2 mM) so that they proposed that inhibition of STK was necessary to permit ketone body activation. Such inhibition they believed might be effected by maintaining a high NTP/NDP ratio. In support of these proposals they noted that various animal tissues able to utilize acetoacetate possessed G-STK activities. They first stated that: "The correspondence between intensive acetoacetate utilization and guanine nucleotide-linked STK is good but not absolute. The corollary that acetoacetate activation by muscle is linked to the inability to use adenine nucleotides, does not appear to hold" (McClellan & Ottaway, 1980). Secondly, in connection with the proposed inhibition of STK during acetoacetate utilization, they cited (Ottaway *et al.*, 1981) mitochondrial matrix values of ATP/ADP ~ 1 and GTP/GDP ~ 100, thus producing the product inhibition of G-STK activity. This proposed inhibition of STK to permit ketone body metabolism, when that very metabolism requires citric acid cycle flux, presents a difficulty which would only be overcome if there were an effective transfer of succinate from OAT to succinate dehydrogenase. However,

our discovery of the existence of two STK enzymes, each specific for its nucleotide substrate, offers a solution. The two enzymes allow metabolic compartmentation of the succinate thiokinase reaction, which enables the supply of succinyl-CoA for ketone body activation with concomitant operation of the citric acid cycle. The results presented in section 5·1 clearly implicate G-STK as being linked to ketone body activation. However, unlike Ottaway who predicted product inhibition of G-STK activity, we discovered elevation in the specific activity of G-STK in tissues characterized by ketone body utilization. It is significant that in normal well fed rat tissues, where the levels of ketone body-utilizing enzymes, OAT and ACT, are greatest, e.g. heart & kidney, (Middleton, 1973 and Beis *et al.*, 1980), the highest levels of G-STK activities were detected. This correlation was also found to be evident when looking at tissues with relatively low levels of OAT and ACT, e.g. brain & skeletal muscle; here, low levels of G-STK were detected. No such connections apparently existed with A-STK activities within rat tissues. Instead of the 10-fold change in G-STK specific activities, A-STK specific activities appeared to be surprisingly constant between rat tissues. The increase in brain ketone body utilization which occurs with elevation of plasma ketone body concentrations allowed measurement of a marked rise in G-STK specific activity, from a very low level to that approaching or surpassing that found in normal heart

tissue. Such changes were not seen in skeletal muscle. Skeletal muscle, although low in OAT, ACT and G-STK activities, is still, due to its relative contribution to body mass (~40%), an important site of peripheral ketone body utilization (Robinson & Williamson, 1980; Beiss *et al.*, 1980). However a number of studies (Beatty *et al.*, 1960, 1964) have shown that ketone body utilization by skeletal muscle was reduced in the diabetic state, but that on addition of insulin skeletal muscle again utilizes ketone bodies and glucose (Balasse & Havel, 1971).

The changes seen in brain G-STK activity upon elevation and reduction of plasma ketone body concentrations induced by diabetes and controlled with insulin, implicate G-STK as supplying succinyl-CoA for the activation of acetoacetate. When this information is correlated with the predicted high mitochondrial GTP/GDP ratio (Ottaway *et al.*, 1981), it would appear that G-STK does indeed catalyse the reaction of succinate to succinyl-CoA utilizing GTP. Much disagreement exists concerning the levels and the ratios of nucleotides within mitochondria. Such evidence that exists will be discussed in section 6·5. What is clear, however, is that G-STK appears to be far from inactive during ketone body activation; rather, it displays elevated specific activities.

6·4·2 Porphyrin biosynthesis

As has been emphasised in section 5·2, the initial

studies of Shemin & co-workers revealed the role and sources of the succinyl-CoA utilized in porphyrin biosynthesis. The results of Shemin and Kumin (1952), using ^{14}C-carboxyl-labelled and ^{14}C-methylene-labelled succinate in the presence and absence of malonate, established that the succinyl-CoA which acted as a precursor of haem, could originate both from oxoglutarate, via normal citric acid cycle activity, and directly from succinate. This result therefore revealed the reversibility of the succinate thiokinase reaction even before the reaction had been fully characterized. Previously, over 20 years ago, Labbé and co-workers (Labbé *et al.*, 1965) demonstrated that in porphyrinogenic liver an elevation in STK activity (~70%) occurred which preceded any increased synthesis of δ-aminolaevulinate and subsequent intermediates of the porphyrin biosynthetic pathway. After our discovery of both G-STK and A-STK enzymes in eukaryotes and identification of an association between G-STK and acetoacetate activation, we repeated Labbé's initial experiment. Examination of hepatic STK activities during porphyria revealed a specific elevation of G-STK, with no change in citrate synthase or A-STK activities. Interestingly, Labbé *et al.* (1965) presented evidence for the existence of two G-STKs in hepatic mitochondria, one constitutive enzyme, unchanged by induction of porphyria, and one that was inducible (3-fold in activity). Labbé & co-workers may have considered the constitutive G-STK as enabling the citric acid cycle to

function. However, a second requirement for succinyl-CoA may exist within the hepatic mitochondrion, besides that required for porphyrin biosynthesis. As discussed earlier in section 5·1·2, specific succinylation and inhibition of the enzyme hydroxymethylglutaryl-CoA synthase may be a regulator for this enzyme (Lowe & Tubbs, 1985) and therefore of ketogenesis. The existence of at least two distinct STKs appears to allow compartmentation of the succinate thiokinase reaction, which in the hepatic and extrahepatic mitochondrion seems to be required to operate in both directions at the same time (citric acid cycle and porphyrin biosynthesis/ketone body activation). To achieve and maintain this bidirectionality, two STKs appear to be present, possessing mutually exclusive nucleotide specificity and control. The G-STK operates in the direction of succinyl-CoA formation and is responsible for the supply of succinyl-CoA for porphyrin biosynthesis and ketone body metabolism, whereas the A-STK enzyme may be involved in maintaining the flux through the citric acid cycle, operating in the direction of succinyl-CoA hydrolysis.

In plant tissue (zea maize) the detection of both A-STK and G-STK within mitochondria may indicate that, although chlorophyll biosynthesis requires glutamate rather than succinyl-CoA as a precursor, the situation may be reversed in the mitochondria. The possibility that the synthesis of haem, required for cytochromes, may occur via succinyl-CoA to form δ-aminolaevulinate, by the action of

low levels of δALA-synthase has been suggested by Fluhr and Harel (1975). However, detection of A-STK and citrate synthase activity within zea maize etioplasts may either result from mitochondrial contamination or represent possible dark phase respiration.

6·4·3 Citric acid cycle

After observing the specific association of G-STK with ketone body activation and porphyrin biosynthesis it was thought that A-STK would be associated with the remaining major metabolic process involving succinyl-CoA, i.e. the citric acid cycle. The bloodstream and procyclic cell forms of *Trypanosoma brucei* provided an ideal system to investigate the involvement of STK within the citric acid cycle. The concerted rise in A-STK, and not G-STK, with other citric acid cycle enzymes upon transformation from bloodstream to procyclic cell forms emphasised again the separate roles of each STK. However, the occurrence of G-STK in *T. brucei* would indicate, in the light of earlier studies, that either ketone body activation or porphyrin biosynthesis was occurring within *T. brucei*. Although the synthesis of porphyrin within the bloodstream form is unlikely, due to an absence of cytochromes (Ryley, 1962), the possibility of such biosynthesis within the procyclic cell forms has not been ruled out (W. Gibson, personal communication). However, the fact that G-STK specific activities were similar in both cell forms probably excludes an association with porphyrin

biosynthesis. In the case of ketone body activation our investigations soon established, for the first time, ketone body utilizing enzymes. It is significant that the natural host for *T. brucei* is not the laboratory rat but rather cattle and other ruminants. Ruminants differ from more simple stomached animals in that there are large rates of alimentary ketone body release into the blood-stream, due to microbial fermentation in the rumen (Heitmann *et al.*, 1987). This means that ruminants, with both alimentary and hepatic ketogenesis, have circulating concentrations of ketone bodies at least 4-5 times those seen in most fed non-ruminants (Williamson & Whitelaw, 1978). The bloodstream form of *T. brucei* thus appears to possess the enzymes required for utilizing the available acetoacetate, probably for biosynthetic purposes. The ketone body utilizing enzymes OAT and ACT were also found in the procyclic form of *T. brucei*, which occurs in the vector, the tsetse fly. There have been limited studies on the importanct of ketone bodies in insects. Acetoacetate is known to increase in concentration in the haemolymph of desert locust (*Schistocerca gregaria*) during flight and starvation. Higher concentrations of ketone bodies have been reported in the haemolymph of the cockroach (*Periplancta americana*) (Candy, 1985). Ketone body utilizing enzymes have also been detected in the nervous tissue of a number of insects (Sugden & Newsholme, 1973; Beis *et al.*, 1980). Bailey *et al.*(1972) have shown that acetoacetate is the principal ketone body in

haemolymph, whereas β-hydroxybutyrate is predominant in the fat body of insects. It is apparent that ketone bodies have the same glucose sparing role in insects as that exhibited in mammals, by supplying energy to nervous tissue and muscles when glucose is scarce. Therefore it is consistent, in view of the association between G-STK and ketone body utilization, to have observed A-STK and G-STK in both bloodstream and procyclic cell forms of *T. brucei*. The slight changes observed in G-STK, OAT and ACT activities, in procyclic cells supplemented with ketone bodies, confirmed this association.

The identification of A-STK as a component of the citric acid cycle in the eukaryotic protozoan *T. brucei* may indicate a wider association throughout nature of A-STK with the citric acid cycle. In rat tissues it is significant that, although A-STK specific activities are relatively constant between tissues, the highest activity is found in heart, which possesses the highest oxidative capacity of all tissues. If A-STK is indeed an integral part of the citric acid cycle, the succinate thiokinase reaction would provide a direct control point for cycle flux that is sensitive to the final product of the cycle, i.e. ATP. A-STK would allow a slowing down of the citric acid cycle when ATP concentrations were high due to product inhibition, yet facilitate citric acid cycle flux at higher ADP concentrations. This mechanism may operate in collaboration with the other regulatory mechanisms that have been proposed for the cycle (Williamson & Cooper, 1980; Rutter & Denton, 1988).

6·5 Final conclusions

6·5·1 *In vivo* enzyme organization

There is growing interest in intracellular enzyme organization, i.e. non-organelle compartmentation of metabolism through organized multienzyme systems. Various interactions between citric acid cycle enzymes have been observed in prokaryotic and eukaryotic organisms (Srere *et al.*, 1987; Beeckmans & Kanarek, 1987), as well as a loosely associated multienzyme cluster (Barnes & Weitzman, 1986). The latter consists of five sequential enzymes, from fumarase to isocitrate dehydrogenase, which elutes from a gel filtration column as a high molecular weight cluster. Such a multienzyme cluster, which can catalyse the sequence of reactions leading from fumarate to oxoglutarate, appears to be capable of catalytic enhancement and/or channelling of intermediates compared with a mixture of the unassociated enzymes.

Along with the molecular and metabolic studies carried out on the eukaryotic STKs, attempts were made to identify any possible physical associations between STKs and other metabolically related enzymes.

G-STK and 3-oxo-acid CoA transferase are known to display limited co-purification (W.A. Bridger, personal communication). This information, together with the strong association between G-STK and acetoacetate activation, prompted the examination of the possible physical association between G-STK and OAT. Gentle osmotic lysis of mitochondria was carried out using a

similar procedure to that successfully used in identification of the high molecular weight cluster of citric acid cycle enzymes (Barnes & Weitzman, 1986). Affinity chromatography was used to study the G-STK/OAT system from Percoll fractionated bovine heart mitochondria, after gentle osmotic lysis. However, no association between G-STK and OAT was observed. In the case of the ultracentrifugation experiments, centrifugation (150,000 x g for 3 h) of osmotically lysed mitochondrial supernatants, which is sufficient to sediment 2-oxoglutarate dehydrogenase, lead to a loss of A-STK activity from the supernatant, while G-STK specific activity increased. However, no A-STK activity could be detected in the pellet. Porpaczy *et al.* (1983) have reported specific association between purified pig heart G-STK and OGDH enzymes, using co-precipitation studies with polyethylene glycol. This may or may not represent the true *in vivo* enzymic organization. The molecular and kinetic similarities that exist between G-STK and A-STK, may reflect a very close resemblance that may exist between the two STKs at the molecular level and confer upon G-STK the potential to interact with OGDH, even though A-STK may be the true *in vivo* partner. Apart from this report of OGDH and G-STK co-precipitation, STK appears to be the only citric acid cycle enzyme which has not been clarified as possessing direct or indirect membrane association. The reason for the absence of association evidence could be that investigators have

concentrated their studies on G-STK, rather than the recently discovered A-STK.

6·5·2 Mitochondrial nucleotides

Due to the absence of a specific GTP/GDP translocation system in the inner mitochondrial membrane, guanine nucleotides (GTP/GDP), unlike adenine nucleotides (ATP/ADP), appear to be trapped within the mitochondrial matrix (Klingenberg & Heldt, 1982). This means that guanine nucleotides are either 'entrapped' during mitochondriogenesis, generated intramitochondrially from adenine nucleotides or transverse the inner mitochondrial membrane, but at rates which short-term experiments cannot detect. Such translocations of guanine nucleotides have been suggested (Söling, 1982) but never demonstrated. In comparison with adenine nucleotides, which have been thoroughly investigated, guanine nucleotides have attracted far less attention. This discrepancy is partially due to the apparent absence of a specific translocation system and also due to the difficulties associated with measurement of guanine nucleotide concentrations. When Ottaway *et al.* (1981) proposed a link between GTP-product inhibition of G-STK and ketone body activation, they claimed that in the mitochondrial matrix, although the ATP/ADP ratio was $\sim$1, the GTP/GDP was as much as 2-orders of magnitude higher. These claims were based on reports (Smith *et al.*, 1974; Williamson *et al.*, 1972) of measurements of GTP/GDP ratios within liver

and heart mitochondria. However, disagreement exists about such a large difference in ratios. There is general agreement that the ratio of ATP/ADP within mitochondria is close to unity, depending upon whether the mitochondria are actively respiring (state 3) or resting (state 4), but in the case of the GTP/GDP ratio differences exist in the estimated values. In the mitochondrial matrix, the GTP-AMP phosphotransferase reaction appears to be the only means of phosphorylating AMP, produced from the activation of short chain fatty acids. From the equilibrium constant of this enzyme and the mitochondrial ADP/AMP ratios observed, a minimum value of the free GTP/GDP ratios can be calculated. When such measurements were made, the evaluated GTP/GDP ratios were demonstrated to be at least 10-times higher than the ATP/ADP ratios (Klingenberg & Heldt, 1982). However GTP/GDP ratios as low as 2·2 have been proposed within liver mitochondria (Siess & Wieland, 1979). At present no accurate method exists for the determination of GDP concentrations within the mitochondrial matrix. It is possible that all nucleotides may be compartmentalized within the matrix, so that the exact ratios (NTP/NDP) *in vivo* may vary greatly from the estimated values.

Ottaway *et al.* (1981) have rightly emphasised that nucleotide diphosphokinase (NDPK), which would be expected to normalize any differences between the two ratios, exists predominantly in the cytosol, with only 5% of the total cellular NDPK activity present in mitochondria.

Moreover, very little of this activity appears to be inside the inner mitochondrial membrane; rather it is located in the intermembranal space (Pedersen, 1973). However, some controversy exists over the absence or presence of NDPK activity within the mitochondrial matrix, a situation further complicated by apparent species and tissue variations (Söling, 1982). If significant amounts of NDPK do indeed occur in the matrix, it would appear that the reaction favours transfer of a phosphoryl group from ATP to GDP. The enzyme from both liver and heart has been shown to be strongly inhibited at ADP concentrations above 50 μM (Colomb *et al.*, 1969, 1972) so that the enzyme would favour GTP formation rather than GTP utilization. These results have largely been overlooked by most biochemical text-books, which confidently predict that the GTP produced by the substrate level phosphorylation step of the citric acid cycle, is used by NDPK to phosphorylate ADP (Stryer, 1988).

6·5·3 Possible origins of GTP & succinate

Our proposal that A-STK is responsible for catalysing the conversion of succinyl-CoA to succinate, resulting in direct production of ATP from the citric acid cycle, poses no problems with respect to the provision of succinyl-CoA or ADP as substrates. However, the proposal that G-STK catalyses the reverse reaction, producing succinyl-CoA from succinate, requires the provision of GTP and succinate.

Intramitochondrial GTP is required for mitochondrial protein synthesis, the regeneration of ADP from AMP and now, in the light of our hypothesis, as a substrate for G-STK. Previously, substrate level phosphorylation was considered to be the main provider of intramitochondrial GTP. However, Krebs & Wiggins (1978) recognised that "an additional GTP-generating mechanism within the mitochondrial matrix must be postulated", to account for the rephosphorylation of AMP, generated by the medium-chain fatty acid thiokinase (butyrate-CoA ligase AMP-forming) enzyme. They suggested that the majority of the GTP formed in the matrix, and utilized in AMP phosphorylation, would originate from NDPK activity. However, another possible source of GTP, would be the enzyme phosphoenolpyruvate carboxykinase (PEPCK). This enzyme catalyses the following reaction:

$$\text{phosphoenolpyruvate} + CO_2 + GDP \rightleftharpoons \text{oxaloacetate} + GTP$$

A tissue specific distribution of PEPCK between the cytosol and mitochondrial matrix is known to occur (Söling, 1982). In the mitochondrial matrix, the direction of the PEPCK reaction has not been clarified. PEP concentrations of $\sim$3 mM have been determined within mitochondria, whereas concentrations of oxaloacetate (OAA) have been calculated to be as low as 1·5 μM (Srere, 1967; Söling, 1982). However, even this quoted OAA concentration may not represent the free OAA concentration within the matrix,

since a number of OAA binding sites exist, e.g. citrate synthase, pyruvate carboxylase, malate dehydrogenase, oxaloacetate decarboxylase, glutamate-oxaloacetate amino-transferase and PEPCK itself. Furthermore, PEP has been found to be a very effective counter ion for citrate exchange across the inner mitochondrial membrane (Robinson, 1971). The incoming PEP, which originates from glycolysis, has therefore been proposed, to be converted into OAA by mitochondrial PEPCK. Such a mechanism would not only provide GTP for the G-STK reaction but also the required precursor (OAA) for succinate formation. After the initial identification that the succinyl-CoA utilized in porphyrin could originate directly from succinate (Shemin & Kumin, 1952) the possible sources of succinate were further investigated. Identification of an elevation in hepatic G-STK activity, upon induction of porphyria (Kurumada & Labbé, 1966), lead to the origin of succinyl-CoA being sought. Kurumada and Labbé (1966) were able to repeat the work of Shemin & Kumin (1952) and went on to identify, through isotope labelling studies, fumarate as the source of succinate. They were also able to demonstrate detection of an NADH-dependent fumarate reductase activity. Thus, there exists the possibility that fumarate arises from the reverse operation of the citric acid cycle enzymes fumarase and malate dehydrogenase, with OAA arising from carboxylation of pyruvate or phosphoenolpyruvate.

In brain mitochondria, where elevation of G-STK

activity accompanies increased ketone body utilization, the operation of the GABA-shunt may possibly provide a source of succinate. However, during activation of acetoacetate by OAT, succinate would be continually regenerated (Fig. 45), while CoA would be removed as acetoacetyl-CoA. Thus for ketone body utilization, the supply of CoA, for G-STK activity, may be more important. The fact that 90% of total cellular CoA occurs within the mitochondria probably would ensure its availability (Idell-Wenger *et al.*, 1978).

6·5·4 Possible compartmentation of STK activities

At present our knowledge of the structural complexity of cells and their organelles is limited. Srere (1981) has indicated that the mitochondrial matrix contains a high concentration of closely packed proteins, so much so that the matrix of mitochondria may be regarded as possessing a semi-solid state with proteins existing almost in a crystalline structure. The consequences of such a model would be to produce microenvironments between the protein/enzyme structures which could contain high concentrations of intermediates even if the overall concentrations were low within the cell. Separate pools of nucleotides and metabolites could also be maintained, as well as the channelling of substrates from one enzyme to the next, along a specific pathway. It may be that such compartmentation of the STKs and/or components of the succinate thiokinase reaction occurs, so that the

succinyl-CoA formed during the operation of the citric acid cycle is distinct and separate from the succinyl-CoA utilized in ketone body activation and/or porphyrin biosynthesis.

6·6 Future work

The STK system provides an excellent model to investigate the factors governing molecular recognition of nucleotides, which are likely to be relevant to other enzyme systems. Comparison of the molecular structures of the nucleotides ADP, GDP and IDP suggests that the substituent groups on C-6 of the purine ring may be important in binding or recognition of the nucleotide to the particular STK. Purification of A-STK and G-STK from a single eukaryotic source will allow direct comparisons of their subunits by proteolytic fragmentation and peptide /amino acid analysis. It may therefore be possible to determine whether the STKs possess similarities between subunits and eventually probe the molecular basis of their nucleotide specificity.

Further work is also required on the kinetic and molecular characterization of the induced liver G-STK during porphyria, and the elevated G-STK from brain during enhanced ketone body utilization. This information together with investigations into the means of regulation of these enzymes (e.g. *de novo* protein synthesis and/or specific activation) will complement the basic molecular comparison studies and may lead, in the long term, to molecular biological studies into the organization and regulation of the expression of the STKs.

Another area of interest worthy of investigation is the possible differential organization and association of the STKs with other metabolically related enzyme systems

within the mitochondria. Techniques which have been used successfully in identifying possible enzyme/enzyme interactions and associations within cells and their organelles, should be employed in respect to both the G-STK and A-STK enzymes.

Finally, although some of the metabolic processes relating to nucleotide specificity are now apparent, the metabolic significance(s) of the molecular size diversity observed between Gram-negative bacteria and other organisms remains uncertain and also merits further investigation.

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APPENDIX

APPENDIX

I Harding's reagent:

Dissolve in order (w/v):
1·2% sodium/potassium tartrate
+ 2% Na_2CO_3 + 2·5% $NaHCO_3$
+ 1·8% potassium oxalate and
store at 4°C.

II Nelson's reagent:

Dissolve 10 g NH_4MoO_4 in
180 ml of distilled water and
slowly add 8·4 ml of conc. H_2SO_4.
10 ml of 12% (w/v) $Na_2HA_5O_4 \cdot 7H_2O$
are then added, and the mixture
made up to 200 ml and stored
at 37°C.

III Tris/hydrazine buffer:

To 1 ml of hydrazine hydrate
add 20 mg EDTA + 2·5 ml 2 M HCl.
Then dilute with 100 mM Tris-HCl
buffer, pH 8·5 to 20 ml.

PUBLICATIONS

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Occurrence of two distinct succinate thiokinases in animal tissues

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Although succinate thiokinase from mammalian sources has hitherto been described as showing substrate specificity for guanine nucleotide, a range of mammalian tissues has here been found to display succinate thiokinase activity with both guanine and adenine nucleotides as substrates. Evidence is presented for the existence of two distinct succinate thiokinases and this is confirmed by their separation by affinity chromatography. Each enzyme is specific for one nucleotide and is inhibited by the non-substrate nucleotide. The physiological roles of the two enzymes is yet to be established.

Adenine nucleotide *Guanine nucleotide* *Nucleotide specificity* *Succinate thiokinase* *Tissue enzyme variation*

1. INTRODUCTION

Succinate thiokinase (succinyl-CoA synthetase) (STK) catalyses the following reversible reaction as part of the citric acid cycle:

$$NDP + P_i + \text{succinyl-CoA} \rightleftharpoons NTP + \text{succinate} + \text{CoA-SH}$$

where NDP and NTP represent nucleoside diphosphate and triphosphate. The STKs which have been most fully studied are those from pig heart and *Escherichia coli* [1,2]. The pig STK was found to function with GDP/GTP as substrates, while the *E. coli* enzyme appeared to be specific for ADP/ATP. These findings led to the established view that animal STKs utilize guanine nucleotides whereas bacterial (and plant) STKs operate with adenine nucleotides, a situation generally presented in textbooks of biochemistry. Some years ago, however, an adenine nucleotide-linked enzyme (A-STK) was reported to occur in blowfly flight muscle [3] and, more recently, A-STK activity has been found in a wider range of animals [4,5], though not in mammals. In some non-mammalian tissues, both A-STK and G-STK activities were detected [4,5] but whether these activities result from a single non-specific enzyme or from the presence of two specific STKs was not resolved.

We have previously reported considerable variation in the nucleotide specificities of a range of bacterial STKs and have shown that some of the bacterial enzymes function as well, or even preferentially, with GDP rather than ADP [6]. In the light of this diversity and the apparent breakdown of the earlier view concerning animal and bacterial STKs it seemed worthwhile to examine carefully the STKs of mammalian tissues for the possible occurrence of both types of STK activity. This communication reports, for the first time, the presence of both A-STK and G-STK activities in mammals and presents clear evidence for their location on two distinct enzyme proteins.

2. EXPERIMENTAL

2.1. *Preparation of tissue extracts*

Fresh beef, pig and sheep tissues were obtained from a local slaughterhouse, immediately cooled on ice and processed in the laboratory within 1 h of slaughter. Rat tissues were taken from freshly

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killed male Wistar rats. Tissues were manually diced into ice-cold 0.1 M Na/K phosphate buffer (pH 8.0) containing 1 mM EDTA, and homogenized with an Ultra-Turrax tissue homogenizer (5 × 15 s bursts interspersed with cooling). After centrifugation (30000 × *g* for 30 min at 4°C) the supernatant solutions were used without further treatment.

2.2. *Preparation of beef heart mitochondrial extract*

Mitochondria were prepared from a whole beef heart as in [7] with the slight modification that they were washed twice in 50 mM Tris-HCl (pH 7.5), 0.25 M sucrose, 1 mM EDTA. The washed mitochondria were resuspended in 20 mM Tris-HCl (pH 7.5), 2 mM $MgCl_2$, and disrupted by ultrasonication (MSE 100 W sonicator operated at 40 W for 6 × 15 s with cooling). After centrifugation (30000 × *g* for 30 min at 4°C) the supernatant solution was used in the experiments reported.

2.3. *Assay of succinate thiokinase*

STK was assayed polarographically [8,9] as described in [6] in the presence of 0.5 mM ADP or GDP.

2.4. *Thermal inactivation*

2.7 ml of 0.1 M Na/K phosphate (pH 8.0), 1 mM EDTA were equilibrated at 48°C. At zero time, 0.3 ml tissue extract was added and aliquots (0.3 ml) were removed at various time intervals and assayed for both A-STK and G-STK activities.

2.5. *Affinity chromatography*

Affinity chromatography was carried out on derivatized GDP immobilized on Sepharose [10]. A column (1.5 × 15 cm) of this affinity material was equilibrated at 4°C with 20 mM Tris-HCl (pH 7.5), 2 mM $MgCl_2$ and 0.5 ml of the beef heart mitochondrial extract (~12 mg protein) was applied. Elution was continued with the equilibration buffer, 1-ml fractions being collected. When the protein content of the eluted fractions had fallen to zero, the elution buffer was changed to include 1 mM GDP. Removal of GDP from the subsequent fractions apparently containing STK was achieved by pooling and gel filtration on a prepacked Sephadex G-25 column (Pharmacia, PD-10).

3. RESULTS AND DISCUSSION

As stated in section 1, all previous studies on mammalian STK have emphasised the specificity of the enzyme for guanine nucleotide substrates (GDP or GTP, depending on the direction of assay of the reaction) and no observation of activity with ADP/ATP has hitherto been reported.

Our first significant finding then is that all the mammalian tissue extracts examined showed STK activity with both ADP and GDP. This was the case with bone marrow and heart from beef, brain, heart, kidney, liver and retina from pig, brain, heart, kidney, liver and skeletal (leg) muscle from rat, and brain and liver from sheep. A single sample of human leg muscle (following hospital amputation) was also found to display activity with both nucleotides. Measurements of STK activity were greatly facilitated by our use of the polarographic method of enzyme assay. We have previously commented [6,11] on the superiority of this assay in comparison with the discontinuous colorimetric hydroxamate method [12] or the spectrophotometric method at 235 nm [13] used by other investigators. Our discovery of STK in cyanobacteria [11], despite its earlier reported absence, owed much to the use of the polarographic assay and the present observations of both A-STK and G-STK activities in mammalian tissues may also have eluded other investigators employing less favourable detection procedures.

The next significant finding was that the ratio of the A-STK and G-STK activities varies between tissues (table 1). A spectrum of ratios was found,

Table 1

Activities of G-STK and A-STK in extracts of various rat tissues

Tissue	Activity (nmol/min per mg protein)		Ratio G-STK/A-STK
	G-STK	A-STK	
Kidney	6.87	1.96	3.5
Liver	4.54	1.5	3.0
Heart	3.58	2.5	1.4
Brain	0.95	1.51	0.6
Skeletal muscle	0.42	1.06	0.4

the G-STK being the predominant enzyme in kidney and liver, more nearly matching the A-STK in heart, and being exceeded by the A-STK in brain and skeletal muscle. It is difficult to reconcile this approx. 10-fold change in the G-STK/A-STK ratio with the presence of a single STK enzyme of loose specificity; one would have to postulate that the 'looseness' of the specificity itself varied between tissues. A simpler explanation is that there are two, nucleotide-specific, STKs whose relative proportions differ between tissues. Ottaway and co-workers [4,5], who first reported A-STK and G-STK in a number of non-mammalian animal tissues, also observed a divergence in the ratio and commented that this may be an indication of the presence of two enzymes, but no further experimental evidence was presented.

The ability of a guanine nucleotide-specific STK to show activity with ADP could result from the additional presence of nucleoside-diphosphate kinase (NDPK) activity and traces of GTP (see [2]). However, the apparent activity of A-STK could never exceed that of G-STK. Thus the fact that A-STK was the major activity in brain and muscle excludes the possibility that A-STK activity results from the action of NDPK. Furthermore, the inclusion of EDTA in the isolation and washing media used in the preparation of beef heart mitochondria has the effect of removing NDPK [5].

The heart mitochondrial preparation also showed the presence of A-STK and G-STK, indicating that both activities are located within the mitochondria. The mammalian G-STK is known

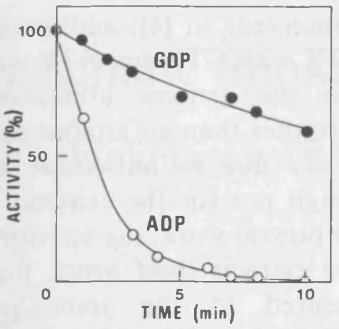

Fig.1. Thermal inactivation of rat heart succinate thiokinases at 48°C. Experimental conditions are described in the text.

to be a dimer of M_r ~70000 [2]. We therefore compared the molecular sizes of the G-STK and A-STK identified in mammalian tissues by means of gel filtration on Sephadex G-200 as in [14]. The two enzyme activities from all the rat tissues listed in table 1 co-eluted on gel filtration, indicating no detectable difference in molecular size between the A-STK and G-STK activities.

One way to test for the presence of two distinct enzymes, as opposed to a single STK with broad specificity, is to compare the activities measured in the presence of ADP and GDP singly with that measured in their joint presence. A single enzyme saturated with the 'preferred' nucleotide would not show increased activity on addition of the second nucleotide. On the other hand, if two enzymes are present, saturation of one of them with its specific nucleotide should still permit the other enzyme to display activity on the addition of the other nucleotide; hence some additivity of activities

Table 2

Specificity of the separated succinate thiokinases and inhibition by the non-substrate nucleotide

Enzyme	Activity (nmol/min per ml)			Inhibition (%)
	0.5 mM ADP	0.5 mM GDP	0.5 mM ADP + 0.5 mM GDP	
A-STK	38.0	0.	24.5	35
G-STK	0	99.5	65.5	34

The two STKs from bovine heart mitochondria were separated by affinity chromatography as described in the text

should be observed. In [4], additivity of the activities of STK with GTP and ATP was used as a criterion for the genuine utilization of both nucleotides (rather than an artefactual display of A-STK activity due to nucleoside diphosphate kinase), though not for the existence of distinct STKs. In the present work, the activities of STK in several tissue extracts (beef heart, pig brain, rat heart) measured in the joint presence of ADP + GDP (both at 0.5 mM, i.e. above saturation) were greater than either of the activities measured in the presence of a single nucleotide. The additivity observed was not strictly the sum of the two activities, but partial additivity (between 50 and 100%) was clearly seen and thus offers further evidence for the existence of two STKs. A possible explanation for the partial additivity is that although each STK is specific for one nucleotide as substrate, the other nucleotide may act as an inhibitor; this point is considered further below.

Additional evidence for the two STKs has been obtained from thermal inactivation studies. Rat heart extract showed the A-STK to be more rapidly inactivated at 48°C than the G-STK (fig.1), supporting the non-identity of the A-STK and G-STK proteins. Similar results were obtained with pig brain.

Finally, conclusive evidence for distinct enzymes was obtained by separating the two activities by affinity chromatography. When the beef heart mitochondrial extract was applied to a column of dialdehyde-GDP-Sepharose (see section 2.5) only the A-STK activity could be detected in the fractions eluted when the column was washed with the equilibration buffer. However, when this buffer solution also contained 1 mM GDP, the G-STK activity was sharply eluted. Neither enzyme showed any activity whatsoever with the other nucleotide. These findings clearly demonstrate the existence of two distinct STKs – one specific for adenine and the other for guanine nucleotide. Table 2 shows that, in each case, the non-substrate nucleotide acts as an inhibitor of the 'other' activity; these inhibitions therefore support the explanation offered above for the observation of only partial additivity when activity measurements were made with crude extracts in the joint presence of both nucleotides.

Our results thus demonstrate that, despite textbook assertions, mammalian cells do possess the enzymic facility to produce ATP directly through citric acid cycle activity. The question of why mitochondria contain two STKs remains to be answered. It is conceivable that they differ in their location or fine level of organisation within the mitochondrion and they may play distinct metabolic roles. It has been suggested [4] that there may be a connection between G-STK activity and ketone body metabolism. Now that we have demonstrated the presence of both G-STK and A-STK in mammals, with tissue-dependent variation, investigations are in progress to explore their particular physiological functions.

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Distinct physiological roles of animal succinate thiokinases

Association of guanine nucleotide-linked succinate thiokinase with ketone body utilization

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Two distinct succinate thiokinases have recently been shown to exist in animal tissues, one specific for guanine nucleotide and the other for adenine nucleotide. Their physiological roles have here been investigated by comparing the levels of the two enzymes in liver and brain of normal and diabetic rats. A marked rise in the level of brain guanine nucleotide-linked succinate thiokinase in the diabetic condition is consistent with an enhanced utilization of ketone bodies and hence with the associated elevated demand for succinyl-CoA for the activation of acetoacetate. Taken together with the reported mitochondrial values of the ATP/ADP and GTP/GDP ratios, the results are interpreted to indicate that the adenine nucleotide-linked enzyme functions as a component of the citric acid cycle whereas the guanine nucleotide-linked enzyme functions in the opposite metabolic direction to produce succinyl-CoA from succinate.

Succinate thiokinase *Guanine nucleotide* *Diabetes* *Streptozotocin* *Ketone body utilization* *(Brain)* *Metabolism*

1. INTRODUCTION

Succinate thiokinase (succinyl-CoA synthetase) (STK) catalyses the reversible reaction:

ADP/GDP + P_i + succinyl-Coa⇌ATP/GTP + succinate + CoA-SH

It is generally presented in textbooks of biochemistry that animal STKs are linked to the guanine nucleotides (G-STK) whereas bacterial and plant STKs operate with the adenine nucleotides (A-STK). We recently reported [1] the existence of distinct A-STK and G-STK enzymes in mammalian tissues with wide variation in the ratio of the two.

The presence of these two STKs poses the question of their physiological roles. One role for STK lies in the citric acid cycle, where it converts succinyl-CoA into succinate. In mammalian tissues, succinyl-CoA also plays a crucial role in ketone body metabolism, activating acetoacetate to acetoacetyl-CoA, with 3-oxoacid CoA-transferase (OAT). To maintain the flux of ketone body utilization, it is necessary to re-cycle the succinate to succinyl-CoA (see fig.1) again using STK. These different demands on the STK-catalysed reaction may well be accommodated by the action of distinct STKs.

Ketone body meteabolism is perturbed in diabetes; the ability to induce diabetic ketoacidosis, e.g. by treatment with streptozotocin [2], thus offers a means of investigating the participation of STK. The results reported here clearly implicate G-STK in ketone body metabolism and,

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as a corollary, we propose that A-STK may function in the citric acid cycle.

2. EXPERIMENTAL

Female Wistar rats (180–190 g) were fed ad libitum. Diabetes was induced by intravenous administration, under anaesthesia, of streptozotocin (150 mg/kg body wt). After 48 h the animals were decapitated; blood samples were deproteinised and glucose concentrations determined as in [3]. Control rats received no treatment. Tissues (groups of 5 rats) were removed immediately and homogenized in 0.1 M Na/K phosphate buffer (pH 8.0) containing 1 mM EDTA using an Ultra-Turrax homogenizer (5 × 15 s bursts with cooling). After centrifugation (30 000 × g for 30 min at 4°C) the supernatants were used without further treatment.

STK activity was assayed as polarographically [4] in the presence of 0.5 mM ADP or GDP. Protein concentrations were determined as in [5].

3. RESULTS AND DISCUSSION

Blood glucose levels in the streptozotocin-treated rats were high (5.38 ± 0.77 mg/ml) in comparison with the control rats (0.61 ± 0.05 mg/ml). These elevated levels indicate the diabetic condition of the treated rats and resemble those found by Schein et al. [2] who also reported a 5-fold rise in ketone body concentration.

Diabetes is accompanied by enhancement of ketone body production by the liver and utilization by the brain [6,7]. We therefore examined liver and brain in diabetic and normal rats. Typical results are shown in table 1. In both tissues, A-STK activity doubled in diabetes. In brain, this may be associated with increased ketone body oxidation via the citric acid cycle; enhanced respiration in liver has also been reported to accompany ketogenesis [8].

The G-STK activity of liver fell in diabetes but that of brain increased strikingly (5- to 10-fold in a series of experiments). As a consequence, the ratios G-STK/A-STK changed very significantly. Whereas in the untreated animals A-STK was the dominant form in brain and G-STK in liver, the reverse was the case in the diabetic rats (table 1).

The ability of the brain to use ketone bodies is not believed to be an enzymic adaptation, as the

Table 1

Effect of diabetes on the levels of succinate thiokinases

Treatment	Tissue	Activity (nmol/min per mg protein)		Ratio G-STK/A-STK
		G-STK	A-STK	
Control	brain	0.9	1.5	0.6
	liver	4.5	1.5	3.0
Streptozotocin	brain	10.0	2.5	4.0
	liver	1.3	3.0	0.43

enzymes responsible for ketone body metabolism (hydroxybutyrate dehydrogenase, 3-oxoacid CoA-transferase and acetoacetyl-CoA thiolase) are present in high activities that are unchanged by diabetes [9].

Changes in the activity of STK in diabetes have not previously been studied, though it has been suggested that the availability of succinyl-CoA could control ketone body utilization [10,11]. Our finding that G-STK increases in brain suggests that it may produce the succinyl-CoA required for ketone body activation. Fig.1 shows the scheme for ketone body activation and subsequent oxidative metabolism via the citric acid cycle. At two points succinyl-CoA is converted to succinate, by reaction with acetoacetate (catalysed by OAT) and by reaction with nucleoside diphosphate and inorganic phosphate (catalysed by STK). In the latter case, the succinate is further oxidised by the later steps of the citric acid cycle to reform oxaloacetate for condensation with the acetyl-CoA produced from the ketone bodies. In the former case, by contrast, the succinate must be reconverted to succinyl-CoA in order to maintain the flow of acetoacetate to acetoacetyl-CoA. This re-cycling could be achieved either by metabolism of succinate round the citric acid cycle, via oxaloacetate and citrate, or by the action of STK operating in the reverse direction.

Ottaway and co-workers [12–14] suggested that ketone body utilisation requires the partitioning of succinyl-CoA (formed by oxoglutarate dehydrogenase) between OAT and STK; as the K_m for succinyl-CoA is very much lower for STK than for OAT, they proposed that inhibition of STK is

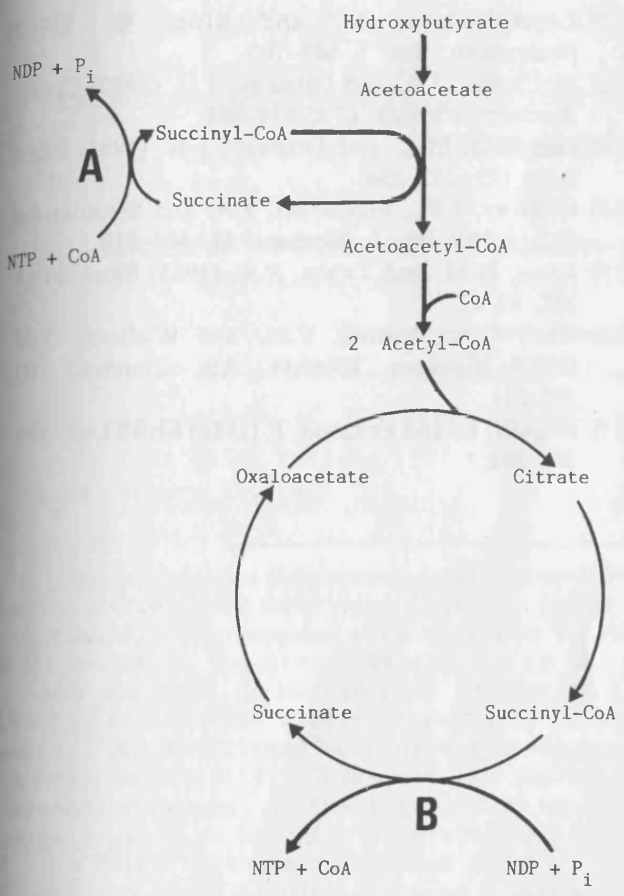

Fig.1. Scheme of ketone body metabolism. NDP and NTP represent nucleoside diphosphate and triphosphate respectively. A and B are two points at which the succinate thiokinase reaction occurs.

necessary to permit ketone body activation. Such inhibition might be effected by maintaining a high NTD/NDP ratio. In support of this proposal they cited [14] mammalian mitochondrial values of ATP/ADP ~1 and GTP/GDP ~100. The proposed inhibition of STK to permit ketone body metabolism, when that very metabolism requires citric acid cycle flux, presents a difficulty, but our discovery of the existence of two STK enzymes, each specific for its nucleotide substrate, offers a solution. The two enzymes allow metabolic compartmentation of the two distinct roles of STK. As part of the citric acid cycle, the A-STK can function to phosphorylate ADP to ATP at an ATP/ADP ratio of ~1, while the G-STK can catalyse the opposite reaction (succinate to succinyl-CoA) at a high GTP/GDP ratio. Our finding that the level of G-STK in brain increases dramatically with increased ketone body utilization supports this proposal and also suggests that re-cycling of succinate to succinyl-CoA is indeed achieved directly by the action of G-STK. It is conceivable that the two STKs are differentially organised within the mitochondrion.

It has been commented above that diabetes leads to increased ketogenesis in the liver. The reduction in the level of liver G-STK which we have observed following streptozotocin treatment may be related to the recent report [15] of the succinylation and inactivation of hydroxymethylglutaryl-CoA synthase by succinyl-CoA. This enzyme functions in ketone body formation; its inactivation by succinyl-CoA may therefore be a control mechanism in ketogenesis. Stimulation of ketogenesis, e.g. by glucagon, appears to be accompanied by reduction in the concentration of succinyl-CoA in the liver [16]; this reduction might lead to de-inhibition of hydroxymethylglutaryl-CoA synthase and hence to increased ketogenesis [15]. Our observation of a decreased level of liver G-STK is therefore consistent with a reduction in succinyl-CoA formation and may be associated with these other processes.

It was recently reported [17] that streptozotocin has a direct inhibitory action on mouse liver G-STK in vitro (50% inhibition at 10 nM streptozotocin), though no in vivo alteration of the activity of this enzyme was observed. We have tested the effect of 100 nM streptozotocin on rat liver and brain G-STK but failed to observe any inhibition. Hence our finding of reduced G-STK in rat liver in vivo following streptozotocin treatment is unlikely to be due to inhibition of activity but rather to a reduction in the level of the enzyme.

In conclusion our results indicate that A-STK and G-STK play distinct metabolic roles within animal tissues. Moreover, as succinyl-CoA is not only involved in citric acid cycle and ketone body metabolism but also acts as a precursor of porphyrins, it may be that STK performs a further function in providing the succinyl-CoA for that biosynthetic process. This is being investigated.

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TWO DISTINCT SUCCINATE THIOKINASES IN BOTH BLOODSTREAM AND PROCYCLIC FORMS OF TRYPANOSOMA BRUCEI

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Two succinate thiokinase activities specific for either adenine or guanine nucleotides have been found in Trypanosoma brucei. Key glycolytic and citric acid cycle enzymes were measured to show repression of glycolysis and derepression of the citric acid cycle in the procyclic form, relative to the bloodstream form. A marked rise in adenine-linked succinate thiokinase activity accompanied a rise in activity of citric acid cycle enzymes. However, guanine-linked succinate thiokinase was found to increase only slightly in activity. These results implicate the adenine-linked enzyme as an essential component of the citric acid cycle, whereas the guanine-linked enzyme appears to be under separate control. This communication also reports for the first time the occurrence of citrate synthase activity in the bloodstream (long slender) form of T.brucei.

Succinate thiokinase (succinyl-CoA synthetase) (STK) catalyses the following reversible reaction:

$$NDP + P_i + \text{succinyl-CoA} \xrightleftharpoons{Mg^{2+}} NTP + \text{succinate} + CoA\text{-}SH$$

where NDP and NTP represent nucleoside diphosphate and triphosphate. Originally, animal tissues were thought to possess only the guanine-linked enzyme (1). However, we have recently reported the occurrence of distinct succinate thiokinases in animal tissues specific for guanine (G-STK) or adenine (A-STK) nucleotides (2).

Subsequent studies (3) have indicated that in vivo A-STK and G-STK may be responsible for the operation of the succinate thiokinase reaction in opposing directions. G-STK in mammalian brain shows a marked elevation in activity with increased ketone body utilization. This supports the proposal that the re-cycling of succinate to succinyl-CoA, required for ketone body activation, is achieved directly by the action of G-STK (3). We therefore

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proposed (3) that the A-STK functions in the energy-conserving role of the citric acid cycle and the present work was undertaken to explore this suggestion further.

During the life cycle of Trypanosoma brucei from mammalian host to insect vector, dramatic changes occur in morphology and metabolism (4). In the mammalian bloodstream T.brucei is exclusively dependent upon glucose for energy, being unable to utilise fatty acids or amino acids due to the absence of a functional citric acid cycle (5). In the insect gut the situation is reversed. Here glucose is scarce and amino acids such as proline constitute the main energy source for the procyclic form (6), proline being oxidized and eventually entering a functional citric acid cycle as oxoglutarate (7).

The bloodstream and procyclic cell forms of T.brucei thus provide an example of a eukaryotic organism in which the enzymes of either glycolysis or the citric acid cycle are repressed or derepressed. We have therefore examined the levels of A-STK and G-STK in both bloodstream and procyclic forms of T. brucei, together with key enzymes of the glycolytic and citric acid cycle pathways. The results reported here clearly identify A-STK with the citric acid cycle whereas a different metabolic role is likely for the G-STK.

METHODS

T.brucei in the bloodstream form were isolated from the blood of 250 g Wistar rats, 71h after injection with 10^7 cells of strain MITat 1.1 (obtained from Dr H.P.Voorheis of Trinity College Dublin) by intraperitoneal injection. The cells were separated from blood components by centrifugation (600 x g) for 10 min at 4°C and further purified on a short column of DEAE-cellulose in isotonic phosphate-buffered saline, pH 8.0, containing 10 mM glucose, as described by Lanham and Godfrey (8). T.brucei in the procyclic cell form (strain EATRO-427, kindly supplied by Dr W.Gibson, Tsetse Fly Research Centre, Langford, Bristol) were cultivated at 26°C on SDM-79 medium (9) supplemented with 10% foetal calf serum. The cells were harvested by centrifugation (600 x g) for 5 min at room temperature and then washed in 100 mM Hepes buffer, pH 7.5, containing 25 mM $NaHCO_3$, 50 mM NaCl, 5 mM L-proline, 5 mM KCl and 5 mM glucose.

Pellets of both bloodstream and procyclic cell forms, after their respective washings, were resuspended in 0.1M Na^+/K^+ phosphate buffer, pH 7.5, containing 20% glycerol and disrupted by ultrasonication (MSE 100W sonicator operated at 40W for 6 x 15 sec with cooling). After centrifugation (5000 x g) for 2 min at room temperature, the supernatant solutions were used without further treatment.

Citrate synthase and succinate thiokinase were assayed polarographically as previously described (10,11). The remaining citric acid cycle and glycolytic enzymes were measured according to Bergmeyer (12). Protein concentrations were determined as in (13).

Table 1. Glycolytic and citric acid cycle enzymes in both bloodstream and procyclic cell forms of T.brucei

Enzyme	Enzyme activities (nmol/min/mg protein)		
	Bloodstream (B)*	Procyclic (P)*	Ratio P/B
Citrate synthase (EC 4.1.3.7)	0.34	2.7	7.9
A-STK (EC 6.2.1.5)	5.4	65	12
G-STK (EC 6.2.1.6)	1.0	1.7	1.7
NADP-Isocitrate dehydrogenase (EC 1.1.1.42)	3.4	28	8.2
Malate dehydrogenase (EC 1.1.1.37)	130	1800	14
Hexokinase (EC 2.7.1.1)	460	14	0.03
Phosphoglucose isomerase (EC 5.3.1.9)	500	74	0.15
Pyruvate kinase (EC 2.7.1.40)	31	9	0.29

*Both freshly harvested or frozen cells, stored at -21°C overnight, yielded similar specific activities for each enzyme. Total cell counts of 1.4×10^{10} and 1.02×10^{10} cells were obtained from bloodstream and procyclic cell forms respectively.

RESULTS AND DISCUSSION

In T.brucei the transformation from mammalian bloodstream to insect gut stage involves dramatic changes in morphology and mitochondrial activity. The long slender bloodstream form appears entirely dependent upon glycolysis for its energy requirements. In contrast, the procyclic form, with active mitochondria, relies mainly on the oxidation of amino acids, citric acid cycle intermediates or fatty acids, resulting in greater versatility and less dependence upon glycolysis. In fact the procyclic cell forms are able to grow even in the absence of free glucose or glucosamine (14).

The typical results, presented in Table 1, demonstrate the enzymic changes associated with the differing metabolic requirements of bloodstream and procyclic forms of T.brucei. The specific activities of hexokinase and phosphoglucose isomerase (both glycosomal) and pyruvate kinase (cytosolic) are repressed in the procyclic cell form. Similar changes have also been reported

by Hart et al. (15). As predicted, the specific activities of citric acid cycle enzymes increase in the procyclic cell form. Interestingly, citrate synthase activity was detected for the first time in the long slender bloodstream form of T.brucei. Succinate dehydrogenase now appears to be the only enzyme of the citric acid cycle absent in the bloodstream form, with the remaining enzymes of the cycle all attenuated (16,17).

The results in Table 1 indicate that the specific activities of the citric acid cycle enzymes increase in concert from bloodstream to procyclic form. Significantly, it is A-STK and not G-STK which displays this marked elevation, thus clearly implicating A-STK as a component of the cycle.

As previously mentioned, studies on A-STK and G-STK from mammalian tissues have shown that one of the roles of G-STK is associated with ketone body activation (3). This, together with predicted high ratios of GTP:GDP in mitochondria (18), implies that G-STK functions in vivo in the direction of succinyl-CoA formation. By contrast, mitochondrial ATP:ADP ratio values are less than or close to 1.0, being as much as 2 orders of magnitude lower than GTP:GDP ratios (18). The A-STK is therefore likely to operate in the direction of succinyl-CoA breakdown coupled to the phosphorylation of ADP to ATP. The results presented in this communication strengthen the suggestion that A-STK functions as an integral part of energy generation by the citric acid cycle.

Our findings concerning STK activities and the first identification of citrate synthase in the long slender bloodstream form of T.brucei were greatly facilitated by our use of the polarographic method of enzyme assay (19). We have previously commented on the superiority of this assay system (10,11) in comparison with other assay methods used for these two enzymes.

ACKNOWLEDGEMENT

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Physiological roles of animal succinate thiokinases

Specific association of the guanine nucleotide-linked enzyme with haem biosynthesis

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The discovery of two distinct succinate thiokinases in mammalian tissues, one (G-STK) specific for GDP/GTP and the other (A-STK) for ADP/ATP, poses the question of their differential metabolic roles. Evidence has suggested that the A-STK functions in the citric acid cycle in the direction of succinyl-CoA breakdown (and ATP formation) whereas one role of the G-STK appears to be the re-cycling of succinate to succinyl-CoA (at the expense of GTP) for the purpose of ketone body activation. A third metabolic participation of succinyl-CoA is in haem biosynthesis. This communication shows that in chemically induced hepatic porphyria, when the demand for succinyl-CoA is increased, it is the level of G-STK only which is elevated, that of A-STK being unaffected. The results implicate G-STK in the provision of succinyl-CoA for haem biosynthesis, a conclusion which is further supported by the observation of a high G-STK/A-STK ratio in bone marrow.

Succinate thiokinase; Guanine nucleotide; Heme synthesis; Porphyrin synthesis; Porphyria; Mitochondrial metabolism

1. INTRODUCTION

Mammalian succinate thiokinase (succinyl-CoA synthetase) (STK) catalyses the following reversible reaction:

$$NTP + succinate + CoA\text{-}SH \rightleftharpoons NDP + P_i + succinyl\text{-}CoA$$

where NTP and NDP represent nucleoside triphosphate and diphosphate. We have recently reported the existence and separation of two distinct STKs in mammalian tissues specific for guanine (G-STK) (EC 6.2.1.6) or adenine (A-STK) (EC 6.2.1.5) nucleotides [1] and have investigated the possible distinct metabolic roles for each enzyme [2,3]. These studies have suggested that, in vivo, A-STK and G-STK may be responsible for catalysing opposing directions of the STK reaction. In mammalian brain G-STK shows a marked elevation in activity with increased ketone body utilization [2], thus supporting the proposal that the re-cycling of succinate to succinyl-CoA, required for ketone body activation, is achieved directly by the action of G-STK. On the other hand, in the eukaryotic parasitic protozoan *Trypanosoma brucei*, during its transformation from bloodstream to procyclic cell form, it is A-STK, not G-STK, which accompanies a concerted rise in the activity of other citric acid cycle enzymes, thus implicating A-STK as an integral component of the citric acid cycle operating in the direction of succinyl-CoA hydrolysis [3].

The other main metabolic role for succinyl-CoA, in the mammalian mitochondrion, apart from its involvement with the citric acid cycle and ketone body activation, is in haem biosynthesis. The first and rate-limiting enzyme of the haem biosynthetic pathway, δ-aminolevulinic acid syn-

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thetase (ALA-S) (EC 2.3.1.37), utilises succinyl-CoA in the following reaction:

succinyl-CoA + glycine ⟶ δ-aminolevulinate + CO_2 + CoA-SH

All mammalian cells contain haemoproteins and possess the capability to synthesize haem [4]. However, the highest rates of haem biosynthesis are located in bone marrow, responsible for erythrogenesis, and in the liver.

There are a number of disease states of haem biosynthesis, collectively known as porphyrias [4]. In hepatic porphyria, perturbation of haem biosynthesis occurs, due to a deficiency or inhibition of an intermediate enzyme. This leads to overproduction and excretion of porphyrin precursors due to derepression of ALA-S synthesis [5]. As a result, the biosynthetic pathway, normally under strict negative feedback control, then operates with elevated levels of ALA-S, thus presenting increased demand for succinyl-CoA. Such demand may be met by an enhanced activity of STK.

Here, we present evidence for the involvement of G-STK in haem biosynthesis. Hepatic porphyria was induced in mice by the drug 3,5-diethoxycarbonyl 1,4-dihydrocollidine (DDC) and the levels of A-STK and G-STK activities were measured. Additionally, bovine bone marrow (sternum) was investigated with respect to A-STK and G-STK activities in view of its essential role as a site for erythrogenesis.

2. EXPERIMENTAL

2.1. *Preparation of bovine bone marrow extracts*

Bovine bone marrow (sternum) was obtained immediately after slaughter. 10 g tissue (possessing a red ossified appearance) was added to 30 ml ice-cold 0.1 M Na/K phosphate buffer (pH 8.0), 1 mM EDTA and homogenized in a Sorvall Omni-mixer (4 × 15-s bursts interspersed with cooling, at full power). After centrifugation (30000 × *g* for 30 min at 4°C) the supernatant solution was used without further treatment.

2.2. *Induction of hepatic porphyria in mice*

Female mice (strain CFLP), each about 30 g and individually housed, were given drinking water ad libitum and starved for 24 h. Each mouse of the treatment group then received 5 g laboratory chow containing 10 mg DDC (kindly supplied by Dr F. De Matteis, MRC Toxicology Unit, Carshalton); the control group received only 5 g laboratory chow. Mice were killed 24 h later and livers were immediately removed. Aliquots (0.5 g) of control and treated livers were retained for total liver porphyrin determination; the remainder of the livers were pooled into control and treated groups for the preparation of mitochondria.

2.3. *Preparation of mouse liver mitochondrial extracts*

Liver tissue, after gall bladder removal, was diced into ice-cold extraction buffer containing 0.25 M sucrose, 50 mM Tris-HCl buffer (pH 8.0), 1 mM EDTA, 0.1 mM EGTA and 0.1% albumin (fatty acid free). Homogenization (5 strokes) in a Potter-type homogenizer (0.3 mm gap) was followed by differential centrifugation, first to remove cell debris (1600 × *g* for 10 min at 4°C) and secondly to pellet mitochondria (30000 × *g* for 25 min at 4°C). Mitochondria were then washed in fresh extraction buffer containing no albumin. Finally, mitochondria were resuspended in 0.1 M Na/K phosphate buffer (pH 8.0) containing 0.05% (v/v) Triton X-100 and disrupted by ultrasonication (MSE 100 W sonicator operated at 40 W for 4 × 15 s with cooling). After centrifugation (30000 × *g* for 30 min at 4°C) the supernatant solution was used without further treatment.

2.4. *Measurement of enzyme activities and protein concentrations*

STK and citrate synthase were measured polarographically [6] as described in [7,8]. STK assays contained 1 mM ADP or GDP. Protein concentrations were determined as in [9].

2.5. *Measurement of total liver porphyrins*

Total liver porphyrins were extracted into 10.2 M perchloric acid/ethanol mixture (1:1) and measured fluorimetrically in an Aminco-Bowman spectrophotofluorimeter using an internal mesoporphyrin standard [10].

3. RESULTS AND DISCUSSION

Porphyric mouse liver, gall bladder contents and prepared liver mitochondria were all characteristically dark red in appearance when compared with controls. The total liver porphyrin content of DDC-treated mice was increased 100-fold, from a control value of 4 nmol/g liver to a DDC-treatment value of 406 nmol/g liver, thus confirming the porphyric state.

The initial and final steps of the haem biosynthetic pathway occur within the mitochondrion [11]. Ferrochelatase (EC 4.99.1.1), the final enzyme of the pathway, catalyses the insertion of ferrous iron into protoporphyrin IX [4]. In the liver of DDC-treated animals, transfer occurs of the 4-methyl group from DDC to one of the pyrrole nitrogens of protoporphyrin IX, converting it to *N*-methylprotoporphyrin [12] which is a potent irreversible inhibitor of ferrochelatase. The decrease in haem production removes the negative feedback control on the system and leads to over-production of ALA-S and subsequently haem precursors [5].

Table 1

Effect of porphyria on citrate synthase and STK activities in mouse liver mitochondria

Treatment	Activity (nmol/min per mg protein)			Ratio G-STK/A-STK
	Citrate synthase	G-STK	A-STK	
Control liver mitochondria	8.9 ± 0.8	3.7 ± 0.4	1.5 ± 0.5	2.5
DDC-treated liver mitochondria	8.7 ± 0.5	7.4 ± 1.1	1.6 ± 0.3	4.6

This increase in ALA-S activity and demand for succinyl-CoA can be seen to be accompanied by an increase in G-STK activity (table 1). Both citrate synthase and A-STK showed no significant change in activities, whereas G-STK doubled in specific activity in the porphyric state compared with the control.

Significantly, bovine bone marrow (sternum) possesses the highest G-STK/A-STK ratio of any mammalian tissue so far examined. Thus, we found the specific activities of G-STK and A-STK to be 2.5 and 0.33 nmol/min per mg protein respectively, giving a ratio of 7.6. This value is considerably greater than the highest ratio reported for any other tissue, i.e. 3.5 for rat kidney [1]. This result, taken together with the sharp increase in mouse liver G-STK in the porphyric state, offers strong support to our proposal that G-STK does indeed supply succinyl-CoA for haem biosynthesis.

Over 20 years ago it was demonstrated that hepatic porphyria is accompanied by an elevation of STK activity [13]. However, at that time it was not known that liver and other mammalian tissues contain both A-STK and G-STK enzymes. Our recent discovery of these two enzymes has made it pertinent to investigate their differential response to the porphyric condition.

This communication, together with the report on G-STK involvement with ketone body activation [3], emphasises the distinct metabolic roles of A-STK and G-STK in mammalian cells. It would seem that in the mitochondrion the STK reaction is required to operate in both directions at the same time (citric acid cycle flux and porphyrin biosynthesis/ketone body activation). To achieve this bidirectionality, two STKs are required, possessing mutually exclusive nucleotide specificity and control.

In conclusion, we propose that A-STK is involved in energy conservation in the citric acid cycle, while G-STK is responsible for the supply of succinyl-CoA for other important metabolic needs. Further studies are currently aimed at exploring the fine level organisation and association of the two enzymes within the mitochondrion.

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Novel nucleotide diversity of succinate thiokinase

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The nucleotide specificity of succinate thiokinase (STK) from a variety of sources has been studied (Palmer & Wedding, 1966; McClellan & Ottaway, 1980; Weitzman & Jaskowska-Hodges, 1982). Gram-negative bacteria can utilize both adenine and guanine nucleotides (ADP/ATP and GDP/GTP) on a single 'large' (tetrameric) enzyme. In contrast, the utilization of these nucleotides by animal STKs occurs on two distinct 'small' (dimeric) enzymes (Weitzman *et al.*, 1986). Gram-positive bacteria were previously thought to contain a single 'small' (dimeric) STK specific for ADP/ATP. However, this communication presents evidence for the existence of a second STK activity in Gram-positive bacteria which is specific for inosine nucleotides (IDP/ITP).

In animal tissues, where both A-STK and G-STK activities are present, I-STK activity can be detected, but in tissues with apparently only A-STK, no I-STK is measurable. Similarly, in Gram-negative bacterial STK, inosine nucleotides may replace their guanine counterparts with similar kinetic characteristics. In Gram-positive bacteria, where no G-STK occurs, an STK specific for inosine nucleotides has been found and this I-STK appears distinct from the A-STK.

Bacteria were grown in nutrient broth at 30°C or 37°C. *Bacillus megaterium* was also grown in minimal salts medium, with 25 mM-succinate, glucose or glutamate as the sole carbon source, to detect any changes in levels of A-STK and I-STK activities. STK activity was assayed polarographically (Weitzman & Jaskowska-Hodges, 1982). Hp.l.c. analysis of all the nucleotides confirmed their purity and hence ruled out the possibility of the detection of activity with a minor impurity. I-STK was detected in the following Gram-positive bacteria: *B. megaterium*, *Bacillus subtilis*, *Staphylococcus aureus*, *Arthrobacter simplex* and *Kurthia zopfii*. Partial additivity which has previously been used as a criterion for distinct enzymes (Weitzman *et al.*, 1986) was observed in the presence of both ADP and IDP at saturating levels (Table 1A). Interestingly, although GDP is not utilized by either enzyme, it appears to inhibit the two STKs differentially (Table 1A). The I-STK shows a greater degree of inhibition by GDP than does the A-STK, and this may be due to the structural similarity between IDP and GDP. In addition, the two STKs exhibit different pH profiles and have different K_m values for their respective nucleotides: K_m (IDP) = 26.7 ± 2.1 μM and K_m (ADP) = 89.4 ± 11.9 μM.

The relative specific activities of the two STKs in *B. megaterium* were found to vary depending upon the stage of growth and also upon the growth medium. On nutrient broth a 10-fold variation in the ratio of A-STK/I-STK occurred between early-logarithmic and late-stationary growth phases, and between different growth media at the same stage of growth (Table 1B). Along with the evidence presented in Table 1A, these results strongly suggest the presence of two distinct nucleotide-specific STKs, the relative proportions of which differ according to physiological conditions. The marked elevation of I-STK activity during growth on succinate may reflect increased formation of succinyl-CoA from succinate under these conditions. The situation in Gram-positive bacteria may therefore resemble that in animal tissues, in which it has been suggested that A-STK may function in the energetic role of the citric acid cycle, whereas the second STK may operate in the opposite metabolic direction, converting succinate to succinyl-CoA for biosynthesis (Jenkins & Weitzman, 1986). Investigations in progress are aimed at further exploration of this possibility.

Abbreviations used: STK, succinate thiokinase; Hp.l.c., high pressure liquid chromatography; NDP, nucleoside diphosphate.

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Table 1. *Succinate thiokinase activities from Bacillus megaterium*

A

Nutrient broth	+ADP	+IDP	+GDP	+(ADP + IDP)	+(ADP + GDP)	% Inhibition	+(IDP + GDP)	% Inhibition
Late-stationary	74.7	16.12	0.0	80.1	58.2	22.2	7.27	54.8

B

Growth media	A-STK	I-STK	Ratio A-STK/I-STK
Nutrient broth			
Early-logarithmic	31.0	0.35	88.57
Mid-logarithmic	73.3	4.8	15.27
Late-stationary	48.74	5.56	8.77
25 mM-Succinate			
Late-stationary	41.09	6.75	6.1
25 mM-Glucose			
Late-stationary	57.9	1.04	55.67
25 mM-Glutamate			
Late-stationary	28.06	0.426	65.87

(NDP = 0.5 mM; all activity values are in nmol/min per mg protein.)

Modification of the regulatory properties of *Acinetobacter* citrate synthase by cross-linking

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Gram-negative bacterial citrate synthases display a diversity of regulatory behaviour depending on the source organism (Weitzman, 1981). The citrate synthase of *Acinetobacter calcoaceticus* is allosterically inhibited by NADH and re-activated by AMP, and it exhibits sigmoidal response curves to both effectors. Additionally, the enzyme is desensitized to NADH inhibition in the presence of 0.2 M-KCl. The interaction of the enzyme with NADH is accompanied by gross conformational changes (Rowe & Weitzman, 1969) and also results in a greatly enhanced thermal stability of enzymic activity. Cross-linking studies (Mitchell & Weitzman, 1983) have been shown to abolish the sigmoidal response to NADH and AMP but to leave the enzyme still sensitive to inhibition and re-activation. The present studies have further extended our previous work.

Cross-linking was achieved by treating the enzyme with the reagent dimethyl 3,3′-dithiobispropionimidate and was confirmed by electrophoretic analysis. Treatment of the cross-linked enzyme with dithiothreitol reversed the process by cleaving the disulphide bonds in the cross-links.

Although the cross-linked enzyme was inhibited by NADH, the ability of KCl to overcome the inhibition was abolished. Cleavage of the cross-links restored the ability of KCl to desensitize the enzyme to NADH. On the other hand, when the enzyme was cross-linked in the presence of KCl the resulting enzyme (tested in the absence of KCl) was insensitive to NADH inhibition. Again, cross-link cleavage restored the sensitivity to NADH.

These results suggest that KCl induces a conformational change in the enzyme which is prevented by the physical constraints of cross-linking. When, however, the cross-linking is undertaken in the presence of KCl, the enzyme is 'fixed' in the desensitized conformation and can no longer respond to NADH. Our earlier electron microscopic study of the enzyme also indicated KCl-induced conformational changes (Rowe & Weitzman, 1969).

Thermal stability studies were carried out on the native and cross-linked forms of the enzyme and also on the cross-linked form after treatment with dithiothreitol. First-order rate constants were determined in the absence and presence of NADH (Table 1). The results show that whereas NADH conferred considerable protection against thermal inactivation of the native enzyme, only slight protection was afforded to the cross-linked enzyme. Cleavage of the cross-links largely restored the protective effect of NADH. Clearly, the conformational changes induced in the enzyme by NADH which enhance thermostability are prevented by the constraints of cross-linking.

These results show that cross-linking may be a valuable experimental tool in the investigation of the conformational changes undergone by citrate synthase in response to its allosteric effectors.

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Table 1. *Effect of NADH on the thermal inactivation of various forms of Acinetobacter calcoaceticus citrate synthase*

With forms (*a*) and (*c*) the temperature was 63°C; with form (*b*) the temperature was lowered to 59°C because of the reduced stability of this form.

Enzyme form	First-order rate constants of thermal inactivation (s^{-1})		Change in rate constant (%)
	No NADH	With 1.8 mM-NADH	
(*a*) Native	6.24	0.32	90
(*b*) Cross-linked	4.47	4.02	10
(*c*) Cross-linked treated with dithiothreitol	4.80	1.26	74

Changes in citric acid cycle enzymes in ageing tomato fruit mitochondria

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Tomato fruit undergo major biochemical changes as they ripen, the most obvious of which are the degradation of chlorophyll and the synthesis of lycopene. Other changes include the rise in CO_2 concentration which just precedes ethylene evolution. Possible routes for the increased CO_2 evolution are either through dark respiration or by stimulation of malate decarboxylation. However, it has never been established whether the flux through the citric acid cycle is responsible for this increase in CO_2 evolution. It is known that mitochondria remain intact and functional during cellular degradation in many fruits (Simpson *et al.*, 1976), and to facilitate degeneration several enzymes are synthesized *de novo* (Tucker & Grierson, 1982). We investigated the specific activities of some citric acid cycle enzymes in intact mitochondria in addition to their total cellular specific activities. We also investigated the enzymes involved in malate decarboxylation in the mitochondria and the cell and finally, we determined whether the specific activities increased in the presence of ethylene.

The specific activities of citrate synthase, malate dehyd-